Studies on nerve growth factor (NGF)-potentiating substances from a brown alga, *Sargassum macrocarpum*

（褐藻ノコギリモク由来の神経栄養因子(NGF)活性促進物質に関する研究）

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CONTENTS

Abbreviations

Abs	Absorbance
AchE	Acetylcholinesterase
AD	Alzheimer’s disease
Akt/PKB	A serine/threonine protein kinase activated by various survival factors
APP	Amyloid protein precursor
ASK	Apoptosis signal-regulating kinase
ASW	Artificial sea water
BFCN	Basal forebrain cholinergic neuron
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
ChAT	Choline acetyltransferase
$CHCl_3$	Chloroform
CNS	Central nervous system
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DQF-COSY	Double quantum filtered ^{1}H-^{1}H-correlation spectroscopy
EDTA	Ethylene diaminetetraacetate
EI-MS	Electron impact-mass spectrometry
ERK	Extracellular signal-regulated kinase (also known as MAP kinase)
FBS	Fetal bovine serum

GRB	A member of guanine nucleotide releasing protein
GSK	A mediator of MAP kinase cascade
GTP	Guanosine triphosphate
HMBC	Heteronuclear multiple bond coherence
HPLC	High performance liquid chromatography
HS	Horse serum
HSQC	Heteronuclear multiple bond coherence
IC_{50}	Concentration that causes 50% inhibition on a specified effect
JNK	c-Jun N-terminal kinases
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MC14	Marine Center compound #14
MC22	Marine Center compound #22
MEK	Mitogen-activated protein kinase kinase
MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser effect spectrometry
P.N.	Proton number
PBS	Phosphate buffered saline

PD	Parkinson’s disease
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
Raf	A serum/threonine protein kinases (also known as MAP kinase-kinase-kinase)
Raf-1	A member of protooncogene cytoplasmic serum/threonine protein kinases of the Raf family
Ras	One of a large family of GTP-binding proteins
R_f value	Rate of flow value
ROS	Reactive oxygen species
RSK	A mediator in MAP kinase cascade
SD	Standard deviation
SHC	Adaptor molecule of the TrkA-initiated signaling cascade
SOS	A member of guanine nucleotide releasing proteins, encoded by *sos* (son-of-sevenless) gene
TLC	Thin layer chromatography
TOCSY	Totally correlated spectroscopy
TrkA	Tyrosine receptor kinase A
UV	Ultraviolet

Abstract

Alzheimer's disease (AD) is anticipated to be a serious social problem since the average human life span is increasing. Although the pathology of AD is still unclear, it has been reported that the deficit of NGF in the brain may be closely related to the cause of AD. A number of *in vivo* animal tests have suggested that NGF has promising potential for the treatment of AD. However, the difficulty of delivering NGF to the brain has become an obstacle in its therapeutic application. Thus, administering of NGF-potentiating substances has been suggested to be an alternative strategy to treat AD.

In this Ph.D. study, two NGF-mediated neurite outgrowth promoting substances, designated as MC14 and MC22, were isolated from a brown alga *Sargassum macrocarpum*. Various aspects of their NGF-potentiating activities, and their mechanisms of action were investigated. Regarding the biological activities, MC14 (sargaquinoic acid) caused a marked enhancement of the NGF-mediated neurite outgrowth from PC12D cells in a dose-dependent manner. MC14-enhanced neurite outgrowth was completely blocked by a mitogen-activated protein kinase (MAP) kinase inhibitor, PD98059, while the promoting effect of MC14 was substantially blocked by protein kinase A inhibitor. However, a protein kinase C inhibitor, chelerythrine chloride did not significantly inhibit the neurite outgrowth promoting effect of MC14. These results demonstrate that MC14 promotes neurite outgrowth via the activation of PKA and MAP kinases-mediated signaling pathway in PC12D cells. Study of its structure-activity relationship indicated that the benzoquinone of MC14 molecule is the structurally essential moiety for the function of MC14. Besides, the substitution of the

benzoquinone at 1’-position with a hydroxy group may significantly enhance its NGF-potentiating activity. Regarding the enzymatic activity, bioassay of the acetylcholin-esterase activity in PC12D cells revealed that treatment of PC12D cells with 3 μg/ml MC14 and 50 ng/ml NGF significantly increased the specific AchE activity by 1.5-fold compared with those treated with 50 ng/ml NGF alone. These results suggest that MC14 promotes morphological as well as biochemical differentiation of PC12D cells. Concerning the neuroprotective effect, MTT measurement indicated that the viability of neuronal PC12D cells decreased substantially in NGF-deprived serum-free medium. However, treatment of cells with MC14 significantly promoted the NGF-induced survival of neuronal PC12D cells in serum-free medium. Approximately 25-40% enhancement of viable cells were detected by the treatment of cells with 1.5 μg/ml MC14 in the presence of 0.02-50 ng/ml NGF, compared with the NGF-only control. Unexpectedly, MC14 alone also exhibited significant neuronal survival supporting effect on neuronal PC12D cells incubated in NGF-deprived serum-free medium. These results imply that MC14 may effectively rescue the neuronal cells in NGF-deficient or even NGF-deprived condition. In addition, MC14 showed protective effect on PC12D cells against hydrogen peroxide-induced oxidative stress, implying that MC14 may protect cells from free radical-mediated cellular damage. Apart from the neuroprotective activity, neurite-regenerating effect of MC14 was analysed by the use of neurite-sheared neuronal PC12D cell model. Microscopic observation showed that the number of neurite-regenerated cells increased from 1.9-fold to 3.6-fold after the neurite-sheared cells were treated with 3 μg/ml MC14 in the presence of NGF at 0.4-50 ng/ml, compared with the MC14-untreated control.

Collectively, the neurite outgrowth enhancing activity, neuronal survival supporting

activity and neurite regeneration promoting activity of MC14 clearly demonstrate that MC14 may be a promising candidate as a therapeutic agent to treat neurodegenerative diseases such as Alzheimer’s disease. On the other hand, MC22 also exhibited neurite outgrowth promoting and survival supporting activities on PC12D cells, although they were less effective than those of MC14.

Chapter I

General Introduction

1. Aging society

The average life expectancy has increased, especially since the turn of 20th century, although the maximum number of years that human beings can live has not increased significantly in the recorded history, In some countries such as Scandinavia and the European Union, the population over age of 60 is projected to rise 30% by the year 2030 (Whitehouse, 1997). In Japan, the birth rate is below replacement levels, so that the ratio of active workers to retirees in the future will cause significantly social, economic and political strains. This increasing tendency of life span has unmasked a new epidemic – dementia, which is defined as the deterioration of mental function.

2. Aging in brain

Several hypotheses have been proposed for the mechanisms of aging, including the DNA mutations, chromosome anomalies, and errors in duplication of DNA (Medvedev, 1972). Another theory, based on the work of Hayflick (1965), has proposed that cells possess a biological clock that dictates their life span. These studies indicate that at least some aspects of aging are intrinsic. No matter what mechanisms exactly cause aging in brain, some general changes occur in the brain with age, such as decrease in brain

weight, decrease in the level of proteins, shrinkage and loss of neurons, changes in cholinergic function, and reduction in enzymes that synthesize dopamine and norepinephrine. These age-related alterations in the brain appear to be normal. However, the age-related neurodegeneration accelerates and intensifies these changes, leading to dementia.

3. Age-related neurodegenerative disorders

(1) Dementia

Dementia is defined as a progressive decline in mental function, memory and acquired intellectual skills. Although dementia is not an inevitable consequence of aging, it is age-related. It is dehumanizing if a patient's intellectual capacity deteriorates to an extent that the performance of routine daily activities is impaired. Several forms of dementia have been distinguished, the most common cause of dementia is Alzheimer's disease.

(2) Alzheimer's disease

Alzheimer's disease (AD) accounts for about 70% of all cases of dementia, and is the leading cause of loss of independent living (Brinton & Yamazaki, 1998). Alzheimer's Disease International estimates that there are currently 12 million AD patients worldwide (http://www.alz.co.uk/alz/index.html). The health care costs for AD in the USA alone have been estimated to be greater than $1000 billion dollars (Whitehouse, 1997). AD is characterized by a progressive loss of cognitive function over a period of 5-15 years before death ultimately occurs (Henderson, 1997). The first symptoms of AD are

deficits in memory function, which increases in severity with increasing duration of the disease. At the later stage, most AD patients will exhibit deficits in higher cognitive functions such as abstract reasoning, judgement and language. Affective disturbances such as apathy, depression, agitation, anxiety or delusion are also apparent (Brinton & Yamazaki, 1998). With the increase in human life span, the number of AD patients is expected to drastically increase because age is the greatest risk factor for this devastating disease.

(3) Pathology of Alzheimer's disease

It is clear that AD is a multifactorial degenerative process (Roses, 1996). About 10% of AD is caused by familiar type. Among them, the mutations in chromosomes 1, 14, 19, and 21 have been identified as an inheritance factor for the disease. The remaining 90% of AD cases is classified as spontaneous AD (Spilantini *et al*., 1998). One of the pathological hallmarks of AD is the formation of beta amyloid plaques, which are accumulations of an insoluble form of beta amyloid protein with 42 amino acids, in the extracellular space in the brain (Crystal, *et al*., 1988). The intracellular characteristic of AD is the neurofibrillary tangles, which are mainly generated by paired helical filaments linked together by hyperphosphorylated tau proteins (Solodkin & Hoesen, 1997). No matter what pathology cause AD, the brain will suffer from loss of synapses and ultimately neuronal death. Thus, the shrinkage of brain and drastic reduction of brain weight are the typical characteristics of AD patients.

(4) Neuronal cell death in AD

Two forms of neuronal cell death have been identified within the central nervous

system (CNS) – necrosis and apoptosis (Altman, 1992). Apoptosis is defined as a process of active cell death, characterized by cell and nuclear shrinkage, chromatin condensation, and membrane blebbing. In contrast, necrosis has been characterized by cellular swelling and lysis resulting in a cytokine-mediated inflammatory response. Besides, necrosis results in the appearance of random DNA fragmentation whereas apoptosis is associated with the cleavage of DNA into fragments with oligonucleosomal-sizes (Altman, 1992; Dragunow & Preston, 1995). Neuronal cell death in AD has been postulated to occur via apoptosis. Recent research has indicated that DNA fragmentation occurs in cells within the temporal cortex and hippocampus of patients with AD (Su *et al*., 1994; Dragunow *et al*., 1995; Anderson *et al*., 1996), further suggesting that apoptosis may be the process of neuronal death observed in AD brain.

4. Pharmaceutical strategies to treat AD

In this decade, various approaches have been immerged, showing promises to treat AD. They include hormone replacement approaches such as estrogen replacement therapy (Birge, 1997); anti-inflammatory approaches such as the use of steroidal and nonsteroidal anti-inflammatory drugs (McGeer & Rogers, 1992); cholinergic pharmaceutic approaches such as the treatment with cholinergic agonists (Bowen, 1981); gene therapies which address the mutations in three genes (amyloid precursor protein, presenilin 1 and 2) (Clark & Goate, 1997); and neurotrophic factor approach (Olson, 1993). In this study, the approach of using neurotrophic factor is concentrated and so it will be described in detail in the following sections.

5. Nerve growth factor and the treatment of Alzheimer's disease

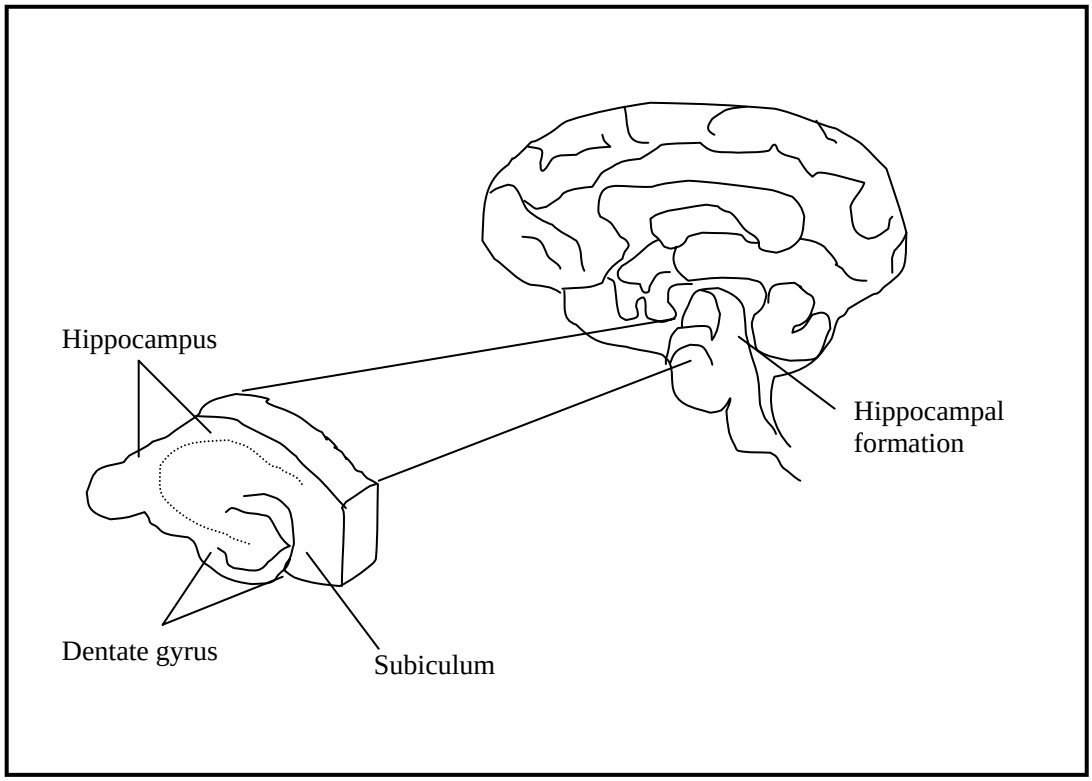
Considerable evidence from both animal and human studies suggests that cholinergic systems are important for learning, momory and cognition (Nicholls *et al*., 1992). Interest in the role of basal forebrain cholinergic neurons in learning and memory has increased after finding that declines in cognitive capacity with aging are paralleled with the loss of cholinergic neurons in the basal forebrain (Dunnett, 1991). This is particularly pronounced in patients with AD. On the other hand, it has been known that NGF can bind to cholinergic neurons of the basal forebrain and prevent their degenerations under certain experimental conditions (Nicholls *et al.*,1992). These led to the suggestion that neuronal degeneration in AD may be prevented or slowed down by the treatment of NGF. The location of hippocampus in the brain and cholinergic innervations of the cortex and hippocampus by neurons in the septal nuclei and nucleus basalis are shown in Fig. 1-1.

6. Physiological functions of NGF

(1) Survival support

For an adult brain, the main role of NGF is to support the survival and maintain the functions of neurons. NGF is produced by the tissues that are innervated by NGF-dependent neurons such as cholinergic, sympathetic and sensory neurons in the CNS. Experimental manipulations have confirmed that the larger the quantity of target tissue, the larger the number of survival neurons (Nicholls *et al*., 1992). *In vitro* studies have shown the NGF-sensitive neurons die if their axons are cut in the adult brain. However,

A



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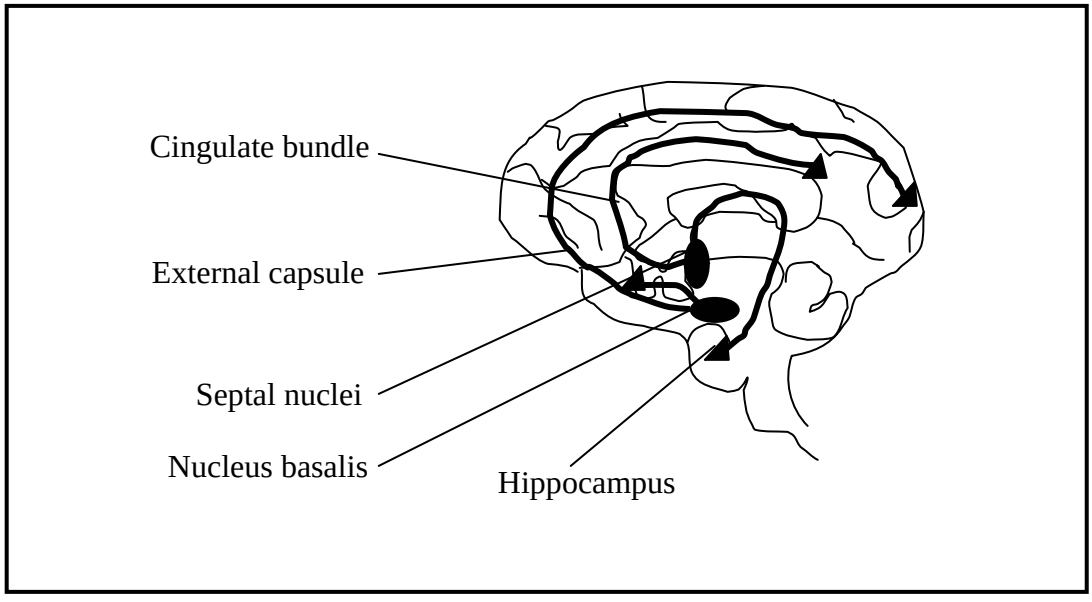


Fig. 1-1. (A) Location of hippocampus in the brain. (B) Cholinergic innervation of the cortex and hippocampus by neurons in the septal nuclei and nucleus basalis (Nicholls et al., 1992).

these neurons survive axotomy if NGF is infused into the CNS (Fischer *et al*., 1989). These results clearly suggest that the survival of these neurons can be directly manipulated by the application of NGF.

One of the most important findings demonstrating the capability of NGF to treat AD is that the basal forebrain cholinergic neurons (BFCNs) respond to and depend on NGF for phenotypic maintenance and neuronal survival (Hefti & Weiner, 1986; Thoenen *et al.*, 1987; Whittemore & Seiger, 1987; Chen *et al*., 1989; Cuello, 1993; Lapchack, 1993). These findings strongly support the potential use of NGF to prevent the cholinergic neuronal loss observed in AD pathology since BFCNs are selectively vulnerable in AD and the impairment of their functions may be responsible for the cognitive deficits observed in AD (Hefti, 1986; Lapchack, 1993). Besides, chronic treatment with NGF has been observed to increase the capacity of surviving hippocampal cholinergic neurons to synthesis, store and release acetylcholine, which is the neurotransmitter that promote nerve functions (Lapchack & Hefti, 1991). On the other hand, one of the well-known neurotrophic action of NGF is that it can protect neurons against axotomy-induced neurodegeneration and aged-related atrophy (Hefti *et al*., 1984). NGF can also protect cultured hippocampal and cortical neurons from excitotoxic injury (Shimohama *et al.*, 1993; Mattson *et al.*, 1995).

Concerning the molecular mechanisms of NGF, it has been proposed that NGF plays a role in its survival supporting action by suppressing the expression of certain ‘suicide genes’, which will induce apoptotic cell death when activated (Estus *et al*., 1994; Ham *et al*., 1995). The loss of transcriptional suppression of the ‘suicide programme’ due to the deficit of NGF level may result in the neuronal atrophy observed in normal aging and the neuronal loss in AD patients. Recent findings have shown that a transcription

factor, nuclear factor κB (NF-κB) may be activated by NGF-mediated intracellular signal to support survival of sympathetic neurons and neuronally differentiated PC12 cells (Taglialatela *et al*., 1997; Maggirwar *et al*., 1998) although the mechanisms by which NF- κB protects neurons from apoptosis remain poorly understood. Previous studies showed a significant induction in the level of the immediate-early-genes, *c-Jun* and *c-Fos* in cultured sympathetic neurons that were destined to die (Estus *et al*., 1994; Ham *et al*., 1995). Therefore, the activation of NF- κB by NGF might suppress the expression of *c-Jun*, leading to the rescue of neurons.

(2) Neuroprotection against oxidative stress

It is known that generation of free radicals causes neuronal cell death. Extensive work has been demonstrated that NGF provides protection from 6-hydroxydopamine, a neurotoxin generates hydrogen peroxide (Castiglioni & Perez, 1981; Perez & Werbach, 1987). The NGF-mediated protection from free radicals generated by direct application of hydrogen peroxide has also been demonstrated. NGF may protect neuronal cells from this peroxidative events and consequent cell death by the induction of free radical detoxifying mechanism such as catalase activity (Jackson *et al*., 1990).

(3) Neural differentiation

The most obvious neurotrophic action of NGF for neural differentiation is its neurite outgrowth inducing effect. If a small piece of a dorsal root ganglion or sympathetic ganglion is placed in a culture dish in a nutrient medium, the neurons only extend short neurites. However, if NGF is added to the medium, the neurite outgrowth of the neurons is strongly stimulated (Mattews, 1998). Apart from inducing neurite outgrowth, NGF

acts as a chemotropic signal for directing the growth of neurite. A classical experiments conduced by Levi-Montalcini (1978) indicated that neurites tend to grow toward regions containing high concentration of NGF.

Induction of neural differentiation by NGF is regulated by the expression of great diversity of gene expressions, leading to the activation of enzymes for the synthesis of neurotransmitter, cellular adhesion molecules and ion channels, and cell cycle arrest (Yan & Ziff, 1997; Li & Wurtman, 1998; Noma *et al*., 1999; Bang *et al*., 2000; Chou *et al*., 2000; Keller & Grover, 2000).

(4) Axonal regeneration

If a neuron is disconnected from its target by severing its axon, a characteristic sequence of changes occurs. As shown in Fig. 1-2, when the axon is severed, the distal portion and a short length of proximal portion of the axon degenerate from the site of lesion. Subsequently, the glial cells will dedifferentiate and phagocytize the axonal remnants. Within a few hours, new axonal sprouts will emerge from near the tip of the proximal stump and begin regenerating. If the neuron successfully reestablishes synaptic contact with a target, the cell body usually regains its original appearance. If the neuron fails to reestablish synaptic contact with its target, the neuron usually shrinks and finally dies. For NGF-sensitive neurons such as sympathetic neurons and sensory neurons, the nearby Schwann cells proliferate and synthesize NGF, which sustain the neurons, stimulate their axonal regeneration, and provide guidance for the navigation of the axonal growth cone to reconnect to their targets (Nicholls *et al*., 1992).

Previous report has described that NGF stimulates the synthesis of structural neural proteins such as growth-associated protein and Tα1 α-tubulin in neuronal cell body

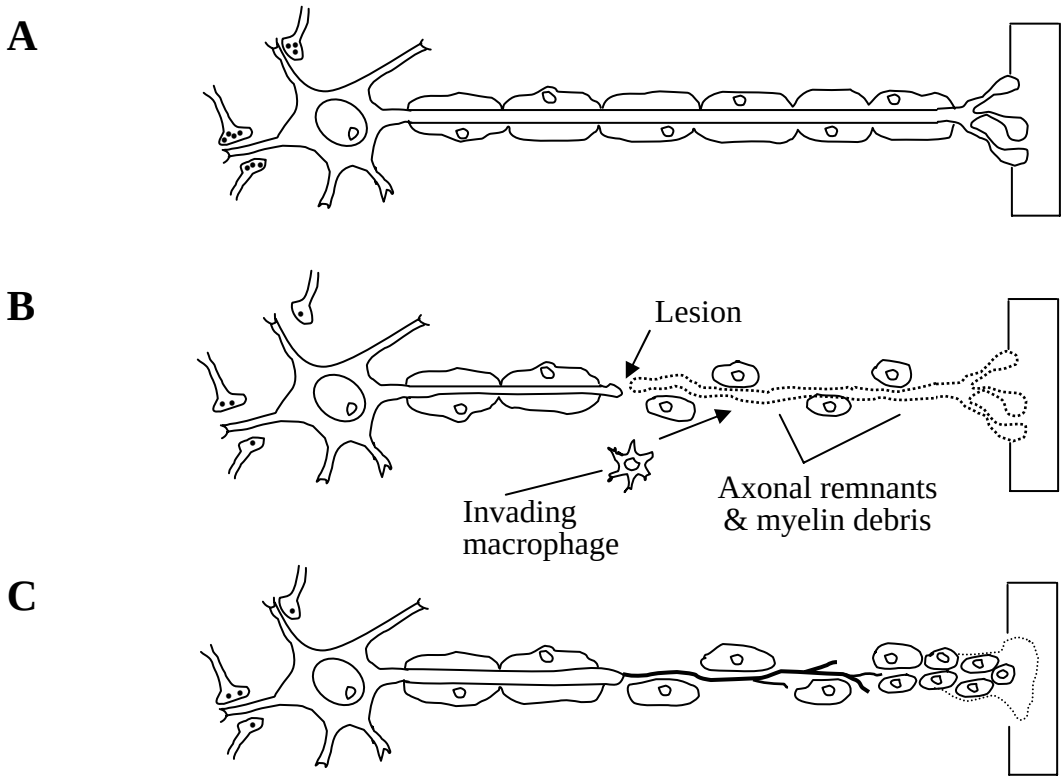


Fig. 1-2. Degenerative changes after axotomy. (A) A typical motor neuron in an adult vertebrate. (B) After axotomy the nerve terminal, the distal segment of the axon, and a short length of the proximal segment of the axon degenerate. Schwann cells dedifferentiation, proliferate, and, together with invading microglia and macrophages, phagocytize the axonal and myelin remnants. The axotomized neuron undersoes chomatolysis, presynaptic terminals retract, and degenerative changes may occur in pre- and postsynaptic cells. (C) The axon regenerations along the column of Schwann cells within the endoneurial tube and sheath of basal lamina that had surrounded the original axon.

(Mohiuddin *et al*., 1995). These cellular alterations induced by NGF are suggested to contribute to the microtubules assembling for the regeneration of neurites (Horner & Gage, 2000). Several animal models with nerve terminals degeneration have shown that administration of human recombinant NGF can facilitate the regeneration of injured sensory neuron (Schicho *et al*., 1999; Horner & Gage, 2000).

7. Experimental evidence for the potential of NGF to treat AD

Given that the vital relationship between NGF and neurons vulnerable in AD, it has been suggested that AD pathology may be due to a deficit of NGF level in the CNS (Hefti, 1986, Hefti & Weiner, 1986; Olson 1993; Semkova & Krieglstein, 1999; Wyman et al., 1999). A great deal of efforts have been put forth to create a strong foundation for the use of NGF to protect cholinergic neurons from death and to restore cholinergic innervation to the hippocampus (Connor & Dragunow, 1998). Substantial studies using cultured neuronal cells and genetic models of neurodegeneration have demonstrated that NGF can effectively prevent neuronal death associated with AD (Connor & Dragunow, 1998). In addition, the animal studies have been very successful. For example, NGF has been shown to significantly ameliorate the neuronal degeneration in rat cerebral cortex and hippocampus after ischemic insults (Shigeno *et al*., 1991; Buchan *et al*.,1997). Effect of NGF in non-human primate brain after fimbrial transection also showed that intraventricular infusion of NGF substantially reduced lesion-induced cholinergic neuronal degeneration, suggesting that NGF may ameliorate memory impairment caused by AD (Tuszynski *et al*., 1991; Koliatsos *et al*., 1991; Burgos *et al*., 1995). On the other hand, Smith *et al*. (1999) reported that NGF gene therapy reverses

age-associated neuronal atrophy occurs in the primate brain. In addition, a genetic mice model of Downs syndrome, which was used for the study of neurodegeneration in AD, has been demonstrated that the atrophy of basal forebrain cholinergic neurons is reversed by NGF administration (Holtzman *et al*., 1993; Holtzman & Mobley, 1994). Taken together, the administration of NGF has been suggested to have great potential to improve cholinergic function and survival, ameliorate age-related impairment in memory and improve cognitive behavior, prevent leison-induced loss of cholinergic neurons, and prevent neuronal loss in hippocampus. More importantly, clinical trails with AD patients have shown promising results. NGF administration early in the disease could potentially much prevent the cholinergic neuronal atrophy observed in the basal forebrain of AD patients (Saffran, 1992; Seiger *et al*., 1993).

8. Limitation of the therapeutic application of NGF

A major obstacle of NGF therapy in humans is its delivery problem. NGF is a large protein molecule (Fig. 1-3) that does not cross the blood-brain barrier (Brinton & Yamazaki, 1998). While intraventricular administration in animals is easily achieved and regulated, it is not the case for AD patients. So far, the administrations of NGF to the human trials are conducted by intracranial or intracerebroventricular infusion of NGF into the patient's brain. As one can expected, it causes great painful to the patient during the process of NGF administration. Therefore, the alternative approaches have been proposed such as the use of NGF-potentiating substance.

9. Alternative strategy: NGF-potentiating substances

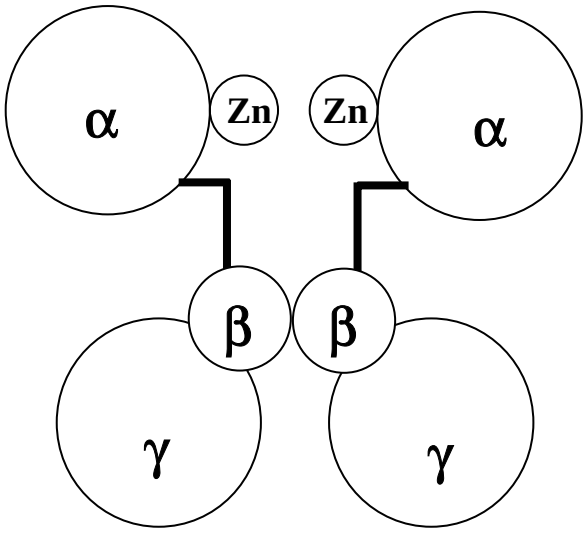


Fig. 1-3. Schematic representation of the molecular structure of NGF. NGF is a high molecular weight complex composed of two α-subunits, β dimer and two γ-subunits. Both α- and γ-subunits have molecular weight of about 26,000 daltons. The β dimer , which possesses all of the NGF activities, is composed of 118 residues with a molecular weight of about 26,512 daltons (adapted from Varon et al., 1982).

As an alternative strategy of addressing the drug delivery problem, it has been suggested that the administration of low molecular weight drugs that can enhance the action of NGF may be a promising approach (Aisen & Davis, 1997; Brinton & Yamazaki, 1998). As mentioned before, AD pathology may be due to the deficit of NGF in the CNS, the administration of such NGF-potentiating drugs may enhance the survival supporting action of NGF to protect the neuronal cells from neurodegeneration. In the literature, the NGF-potentiating substances have shown promising enhancing effect on NGF. Several synthetic compounds such as AIT-082, SR57749A and Aroclor 1254 have been reported to show NGF-potential effect in vitro (Angus *et al*., 1995; Middlemiss *et al*., 1995; Pradines *et al*., 1995). Apart from synthetic compounds, a number of naturally occurring substances are also found to be the NGF-enhancers. They will be described in the section 10.

10. Sources of NGF-potentiating substances

A number of natural compounds that exhibit NGF-enhancing effects have been successfully isolated from Fungus, medical plants, chinese mushroom, coffee bean, marine sponge and the rat basophilic leukemia cells (Suzuki *et al*., 1998; Ito *et al*., 1999; Li *et al*., 1999; Tohda *et al*., 1999; Cheung *et al*., 2000; Li *et al*., 2000). These findings demonstrate that NGF-potential substances can exist in diverse sources. In our laboratory, we have been focusing on searching for the NGF-potentiating substances from the marine algae because they have been demonstrated to be the potential source of unique and pharmacologically useful compounds (Table 1-1). A screening program

Table 1-1. Pharmacologically useful compounds from algae.

Compound	**Source**	**Use**
Fucosterol	*Phaeophyta*	Base for sexual hormone
Sterols	*Fucus gardneri* *Sargarrum muticum*	Reduction in blood cholesterol levels
Caulerpin	*Caulerpa spp.*	Mild anaesthetics
Caulerpicin	*Caulerpa spp.*	Mild anaesthetics
Pachydictyol	*Pachydictyon coriaceum*	Fungicidal/bacteriocidal activity
Isozonarol/Zonarol	*Dictyopteris zonaroides*	
Squalene	*Fucus vesiculosus*	
Stypoldione	*Stypopodium zonale*	Antitumour properties

(Adapted from Chapman, 1979)

of the marine algae had been conducted in our laboratory from 1994-95 (Sagara *et al*., 1997). The bioassay of neurite-outgrowth promoting activity on PC12D cells, a subline of PC12 cells, was used for the screening of algae. The screening result will be described in section 13. As the PC12 cell line is also a useful model for studying other biological effects of NGF on neuronal cells, it will be described in more detail in the following section.

11. PC12 cell line: A versatile cell model for studying the actions of NGF on neuronal cells

(1) Origin of PC12 cell line

Over the past decades, the clonal PC12 cell line has been widely used for various model studies on neurons (Greene & Tischler, 1976). PC12 cell line was originally cloned in 1975 from the transplantable rat adrenal medullary pheochromocytoma (a tumors of the chromaffin cells). PC12 cells share many common properties as sympathetic neurons such as responsiveness to NGF, storing and secreting catecholamines (Greene & Tischler, 1976). It has been suggested that PC12 cells and sympathetic neurons develop from the same embryological origin, therefore, PC12 cells possess the pluripotency of a primitive progenitor which can differentiate along the lines of either chromaffin cells or sympathetic neurons, with NGF promoting their differentiation in a neuronal direction (Greene and Tischler, 1976).

(2) Morphological differentiation in response to NGF

A major characteristic of PC12 cells is that they respond to NGF by slowly shifting

from a proliferative pheochromocytoma cell-like phenotype (naïve PC12 cells) to that of nonproliferating, neurite-bearing sympathetic-like neuron (neuronal PC12 cells). This specific feature of PC12 cells has made them an excellent cell model for the study of the mechanisms of action of NGF and the role of NGF in developmental neuroscience. In addition, the neurite outgrowth response of PC12 cells to NGF provides a useful morphological parameter for bioassay of the effect of NGF and other substances that can promote the action of NGF (Greene *et al*., 1987).

(3) Biochemical differentiation

PC12 cells are also useful in neurochemical study because they synthesize and store the catecholamine neurotransmitters (dopamine and norepinephrine). The levels of catecholamine and their synthetic enzymes are comparable to those found in rat adrenals (Greene and Tischler, 1976). Besides, it has been found that the acetylcholinesterase and choline acetyltransferase activities in PC12 cells can be regulated by NGF. The former enzyme has a specific activity that does not vary as a function of cell density (Rieger *et al*., 1980). Thus, the measurement of acetylcholinesterase in PC12D cells has become a biochemical marker for the action of NGF (Greene & Rukenstein, 1981).

(4) Apoptotic cell death

The rapid advancement about the understanding of molecular mechanisms involved in neuronal cell death and other neurodegeneration processes including AD may be mostly contributed by the research using PC12 cells as neuronal cell death model. Neuronal PC12 cells differ from sympathetic neurons in that they can survive without

NGF if cultured in the presence of serum (Green & Tischler, 1976). However, neuronal PC12 cells rapidly die in serum-free nutrient unless NGF is present (Green, 1978). In other words, the neuronally differentiated PC12 cells depend on NGF for their survival in serum free medium. The utilization of neuronal PC12 cells in this serum-free paradigm has been extensively used to elucidate the mechanisms by which neuronal survival and cell death are regulated by NGF. Recent researches have proved that this model is also suitable for investigation of the apoptosis mechanisms. Substantial evidence has shown that the death of NGF-deprived neuronally differentiated PC12 cells resembles apoptosis in neurons, including the requirement of new gene expression, possibility of cell death blockage by transcription inhibitors, and the DNA fragmentation (Batistatou & Greene, 1991; Mesner *et al*., 1992; Mesner, 1995). These features of neuronal PC12 cells establish them as one of the most important model for studying neuronal apoptosis.

(5) PC12D cells

PC12D cells were first isolated from the PC12 cell cultures by Dr. Sano *et al* (Institute for Developmental Research, Aichi Prefecture Colony, Japan). They found that PC12D cells extend neurites in response to NGF more rapidly than the PC12 cells. Neurite outgrowth is observed on PC12D cells within 24 h in response to NGF (Katoh-Semba *et al*., 1987) while that on PC12 cells takes about 6-7 days (Greene & Tischler, 1976). Therefore, PC12D cells are ideal for screening of large amount of samples. Regarding the enzymatic activity, the tyrosine hydroxylase, acetylcholinesterase, and ornithine decarboxylase activities in PC12D cells can be enhanced by NGF (Katoh-Semba *et al*., 1987), suggesting that the biochemical events

of PC12D cells are similar to those of PC12 cells.

12. Signaling pathways regulating the cellular responses

(1) Protein kinases and signal transduction

Phosphorylation and dephosphorylation of cellular proteins provide a molecular switch for activating or deactivating the intracellular proteins. Reversible protein phosphorylation is mainly manipulated by various protein kinases. Phosphorylation by a kinase results in the activation (or sometimes deactivation) of its substrate, which can be another kinase or other downstream effector protein. In this way, the intracellular signal can be transmitted from the cell surface into nucleus, where the gene transcriptions are initiated for various cellular responses such as differentiation, cell division or apoptosis (Bruce *et al*., 1994).

(2) Classification of kinases

Protein kinases can be classified based on their regulatory mechanisms. For example, protein kinase A (PKA) is activated by the changes in the concentration of intracellular second messengers (e.g. cyclic AMP, cyclic GMP, and inositol triphosphate). The common feature of this class of protein kinases is that they are inactive in the absence of second messengers. On the other hand, tyrosine receptor kinase A (TrkA) represents another group of kinases, which contain receptor domain that can be activated by ligand-binding via autophosphorylation (Bruce et al., 1994). Other class of kinases can be activated by other kinase, such as the mitogen-activated protein (MAP) kinase (Hardi & Hanks, 1995).

(3) Role of kinases in signaling transduction network

Intracellular signaling pathways form a complex networks that allow the interconnect systems of proteins to regulate specific cellular responses. If gene expression is involved, signal has to transmit from cytoplasm to nucleus. This job is usually carried out by a member of the MAP kinases. There are large number of distinct upstream MAP kinase and MAP kinase kinase to provide a cascade that can be amplified from the surface signals in order to increase the sensitivity of the responses. Recent findings have indicated that several MAPK cascades exist that are activated by different external signals (Iwasaki *et al*., 1999; Klockow *et al*., 2000; Pouyssegur, 2000). It should be noted that cross talk between kinases in the MAP kinase-dependent pathways is possible in order to modulate the cellular responses. Other kinases, such as protein kinase C (PKC) are believed to coordinate the signaling networks because PKC can phosphorylate certain upstream members of the MAP kinase family (Bruce *et al*., 1994). In addition, several mediators in the MAP kinase cascades have been identified, such as PKC, Raf-1, MEK, ERK, RSK, and GSK3. ERK1 and ERK2 are the first MAPK isoforms to be identified in the MAPK family. Following their activation, the ERKs phosphorylate a large number of regulatory proteins and thus controlling several cellular processes including transcription, translation, and cytoskeletal rearrangement as the nuclear targets of activated kinases are believed to be transcriptional activation factors. Actually, the signaling pathways have been implicated in the regulation of a wide variety of cellular processes such as proliferation, differentiation, development, cell cycle, and programme cell death (Bruce *et al*., 1994).

(4) Signaling pathway involved in NGF-induced neurite outgrowth on PC12 cells

Although the mechanisms underlying neuronal cell differentiation in detail have not been fully understood yet, the signaling pathways induced by NGF leading to neurite outgrowth on PC12 cells have been characterized. In response to the stimulation of cells with NGF, the tyrosine receptor kinase A is activated by autophosphorylation on tyrosine resides. The autophosphorylation of TrkA results in the activation of a serious of adapter molecules including SHC, GRB, and SOS by protein-protein interactions. They activate multiple downstream effector proteins such as a small GTP binding protein, Ras, which in trun activates Ras-MAP kinase signaling cascade via MEK. The activated MAP kinase translocates into nucleus and activates Elk-1-dependent gene transcription, which initiates neurite outgrowth response in PC12 cells (Yoon *et al*., 1997; Encinas *et al*., 1999). It has been reported that other pathways can also be involved in neurite outgrowth and they seem to be NGF-independent. These signaling pathways include cyclic AMP-mediated pathway or PKC-dependent pathways. Some cross-talk between the signal cascades may exist although most of these signals are transmitted via sequential activation of cytoplasmic protein kinases (Matthews, 1998). As described above, the intracellular signaling systems activated by NGF are complicated, and other cellular responses induced by NGF have not been fully elucidated. This complexity is expected for activating a wide variety of genes in the proper sequence to produce a functional neuron (Matthews, 1998).

(5) Signaling molecule inhibitors

Various signaling molecular inhibitors for each class of kinase have been widely

used to investigate the functions of certain kinases, and to identify their corresponding signaling pathways in regulating neuronal cell processes in response to external stimuli or neurotrophic factors (Felipo *et al*., 1990; Pawlowska *et al*., 1993; Pang *et al*., 1995). Specific inhibitors can act on both catalytic and regulatory domains of target kinases to prevent substrate interaction, or to block the ATPase activity. For example , a PKC inhibitor, chelerythrine chloride can bind to the active site of the kinase while another PKC inhibitor, calphostin C inhibits the cofactor binding (Felipo *et al*., 1990). Some inhibitors are non-specific as they can inhibit a number of different protein kinases or similar isozymes. For example, K252a can inhibit general intracellular protein kinases and it can also specifically inhibit TrkA receptor at a much lower effective concentration for general kinase inhibiton (Pawlowska *et al*., 1993).

13. Research objectives

The screening of a total number of 299 algal species from 598 collected samples has led to the identification of a neural active compound, which was designated as MC14, from a marine brown alga, *Sargassum macrocarpum* collected from Kashiwa island, Saga, Japan (Fig. 1-4). Bioassay result indicated that MC14 significantly enhances the NGF-induced neurite outgrowth from PC12D cells (Sagara & Kamei, 1998).

In this doctoral thesis research, the primary objective is to elucidate the chemical structure of MC14. The modified procedure for the purification, and the results of chemical structure analysis of MC14 will be described in chapter II.

In chapter III, the distribution of MC14 in the algal body of *S. macrocarpum* was studied for exploring the possibility of simplifying the original purification procedure of

MC14.

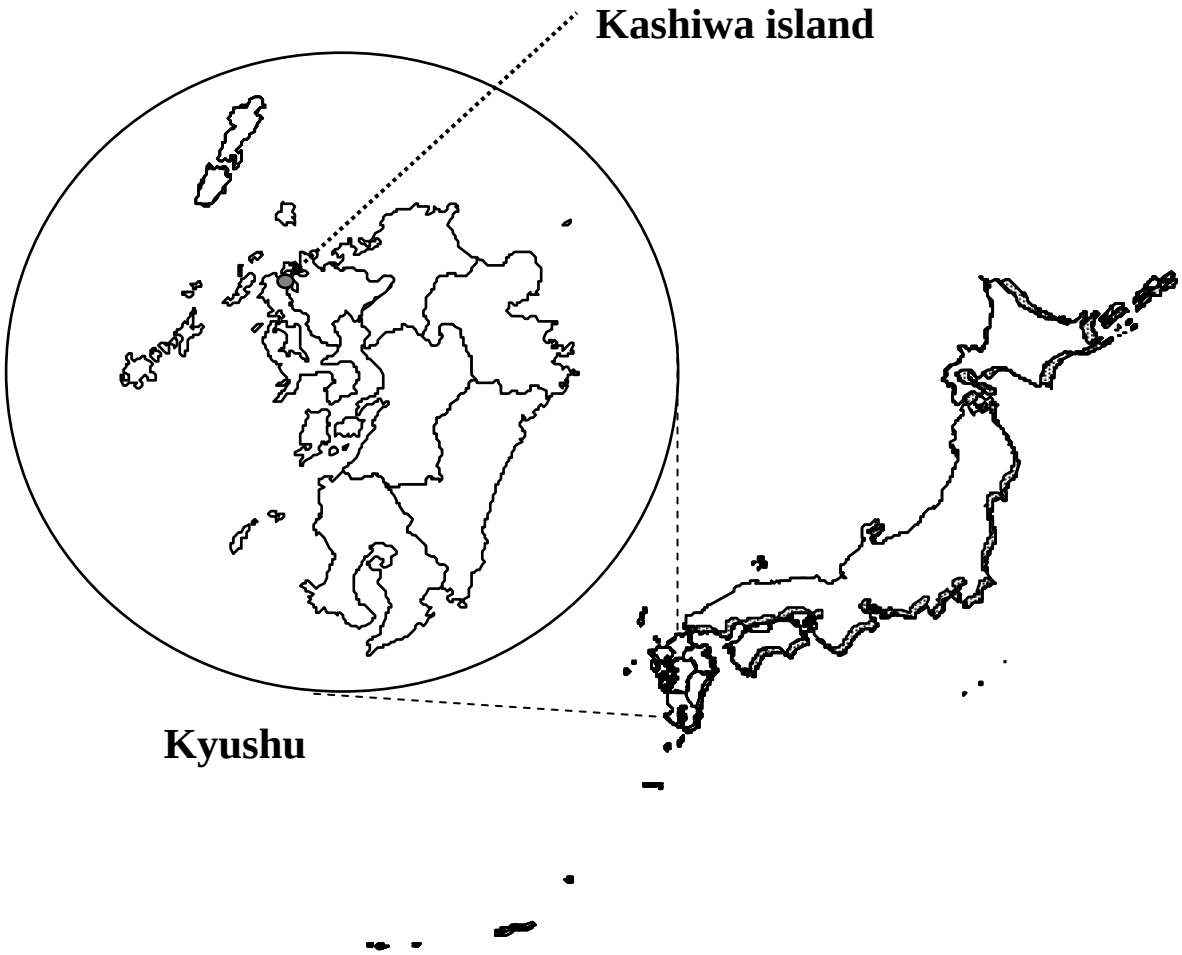


Fig. 1-4. Sampling location ( ) of the brown alga, *Sargassum macrocarpum.*

Since the NGF-induced neurite outgrowth on PC12D cells is the most obvious indication of NGF's action, the NGF-potentiating activity of MC14 was first evaluated by examining its NGF-mediated neurite outgrowth promoting activity on PC12D cells. Apart from serving as the first evidence of its NGF-potentiating activity, the neurite outgrowth promoting effect of MC14 is also an importing implication for its capability of promoting neural differentiation during embryonic developmental stage. Therefore, this activity was studied in detail, including the mechanisms of action of MC14 in the intracellular signaling pathways. The elucidation of signaling pathway mediating the MC14-enhanced neurite outgrowth would be important for exogenous manipulation of neuronal differentiation. In addition, these results would be useful for understanding the regulatory mechanisms leading to this important cellular response during neurogenesis. For confirming the use of PC12D cells as important cell model for studying neuroscience, the mechanisms of NGF-induce neurite outgrowth in PC12D cells were also investigated and these results will be described in chapter IV. On the other hand, a biochemical marker, acetylcholinesterase activity was measured in PC12D cells to investigate the functional differentiation promoting activity of MC14.

As described in chapter I, NGF has diverse neuronal actions, including the induction of neural differentiation, neuronal survival support, neuroprotection and neuroregeneration. Besides, substantial evidence has indicated that these effects may be mediated by different mechanisms via distinct signaling pathways. Therefore, it is important to further clarify whether MC14 can promote these neuroprotective effects of NGF.

Given that the ultimate objective of this study is to evaluate the potential of MC14 as a therapeutic agent to treat neurodegenerative diseases, it is essential to investigate

the neuronal survival supporting activity of MC14. In chapter VI, the survival supporting effect of MC14, as well as the NGF-induced survival promoting effect of MC14 will be described. To get a further insight into its neuroprotective effect, the cellular mechanisms regulating this effect of MC14 on neuronal PC12D cells were investigated. Furthermore, as a number of neurodegeneration is resulted from the oxidative stress such as the beta-amyloid accumulation in the brain of AD patients, the neuroprotective effect of MC14 against oxidative stress was studied. These result will be discussed in chapter VI.

On the other hand, synaptic connection has important role in normal function of nervous system, it has been demonstrated that synapses are usually the first site to be affected in neurodegenerative diseases. For investigating whether MC14 can help restoring the functional regeneration of synapses, the neurite regenerating effect of MC14 was studied in various aspects, including the neurite degeneration alleviating activity, neurite regeneration effect under NGF-deficient condition. Moreover, neurite-severed neuronal PC12D cells were used to examine the neurite regrowth activity of MC14. These results will be discussed in chapter VII.

To analyze the structure-activity relationship of MC14, various structural analogs of MC14 were examined for their NGF-induced neurite outgrowth activity. The result will be described in chapter VIII. During the comparative study of MC14 analog, vitamin K compounds were found to share structural similarity to MC14. To explore the activity of vitamin K in the nervous system, the NGF-potentiating activity of vitamin K compounds and their mechanisms were studies, and will be described in chapter IX.

During a modified procedure for the extraction of MC14, another NGF-potentiating substance were identified from the same alga, *S. macrocarpum*. This substance has been

successfully purified and its chemical structure was elucidated. The purification procedure, together with the results of NGF-potentiating bioassays will be described in chapter X.

Chapter II

Isolation and Purification of MC14 from *Sargassum macrocarpum*

In general, the isolation of natural products is usually achieved by solvent extraction, open column chromatography, thin layer chromatography (TLC), and finally purified by high performance liquid chromatography (HPLC). Every natural product isolation is different and a targeted substance can be isolated and purified by various methods. As mentioned in chapter one, the previous algal extraction procedure consumed a relatively large amount of chloroform at the initial solvent extraction step. Considering the higher cost and high toxicity of chloroform, the isolation procedure has been modified in order to extract MC14 from the crude algal extract in a more effective and safer way. In this chapter, the detailed modified procedure for the isolation and purification of MC14, its physical characteristics and the structural determination of MC14 will be described.

2-1. Materials and Methods

2-1-1. Preparation of crude extracts

The algal sample (500 g wet weight) of the brown alga *Sargassum macrocarpum* was washed 3 times with artificial sea water (ASW, Jamarine Laboratory) to remove the sands and other debris attached on the algal surface, and once with phosphate buffered saline (PBS). The sample was then cut into small pieces and homogenized in 2L PBS

with a domestic mixer. The residue after centrifugation at 5000 x g was extracted with 2L methanol (MeOH) for 1h by a mechanical homogenizer (Polytron, Kinematica) and the MeOH-extract was obtained after suction filtration through a #1 filter paper (Advantec). The resultant residue was further extracted with 1L chloroform ($CHCl_3$). The filtrates of MeOH- and $CHCl_3$-extracts were pooled together and concentrated by vacuum-evaporation to minimal volume, and then partitioned with 1L hexane-water-methanol (3:2:1) mixture. The fraction of aqueous methanol layer was further partitioned with chloroform-methanol-water (3:1:2). The chloroform fraction was subjected to the silica gel column chromatography.

2-1-2. Silica gel column chromatography

The chloroform-layer collected from solvent extraction described above was concentrated to suitable volume and chromatographed on silica gel (silica gel 60, Merck) (column size, ∅ 3.2 x 50 cm) eluting with chloroform-methanol (15:1). The flow rate was adjusted to 5ml/min and a volume of approximately 5 ml per fraction was collected in glass tubes. The active fraction in the following purification step was determined in the same way.

2-1-3. Size exclusion chromatography

The pooled active fractions obtained from the silica gel open column described above were subjected to the size exclusion chromatography (Toyopearl HW-40F, TOSOH) (column size, ∅ 3.2 x 50 cm1.7 x 50 cm) with 100% MeOH as eluting solvent. Active fractions were pooled, evaporated to suitable volume and then redissolved in chloroform-methanol (98:2).

2-1-4. High performance liquid chromatography (HPLC)

The active factions collected after size exclusion chromatography were purified by HPLC using Inertsil SIL column (∅ 20 x 250 mm, GL Science), eluting with chloroform-methanol (98:2) at a flow rate of 5 ml/min and detecting at 250 nm by a UV detector (UV-970, Jasco). The purified active substance MC14 was confirmed its purity by Inertsil SIL HPLC column (∅ 7.6 x 250 mm, GL Science) eluting with chloroform-methanol (98:2) at a flow rate of 0.5 ml/min and detecting at 250 nm by a UV detector as described above.

2-1-5. UV/VIS absorption spectrometry

The UV/VIS spectrum of the purified MC14 was determined in MeOH by a spectrophotometer (V-550, Jasco).

2-1-6. Mass spectrometry

The molecular weight of MC14 was further confirmed by high resolution electron impact mass spectrometry (EI-MS, JMS-DX303, JEOL).

2-1-7. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectrometries

The purified MC14 was measured to obtain ^{1}H - and ^{13}C-nuclear magnetic resonance spectra by Toray Research Center. The ^{1}H - and ^{13}C-NMR spectra of MC14 were recorded in CD_3OD by a GSX400 NMR spectrometer and a UNITY INOVA600 NMR spectrometer (Varian), respectively.

2-1-8. Bioassay of neurite outgrowth promoting activity

PC12D cells were harvested and seeded at a cell density of 5 X 10^3 cells per well on 96-well tissue culture plate. After 24 h incubation in humidified air (5% CO_2) at 37° C in DMEM supplemented with 10% horse serum and 5 % fetal bovine serum, MC14 and 10 ng/ml NGF were added to the cells. Cells treated with 10 ng/ml and 50 ng/ml NGF alone were assigned as negative and positive controls, respectively. After 48 h incubation, the neurite outgrowth promoting activity was determined as follow:

$$\text{Neurite outgrowth Promoting activity} = \frac{\text{Number of neurite-bearing cells}}{\text{Total number of cells in the same field of observation}}$$

Neurite-bearing cells were defined as cells with processes twice longer than the diameter of their cell bodies. The activity of the sample was compared with 10 ng/ml NGF (negative control) and 50 ng/ml NGF (positive control)-treated cultures. For each datum point, the mean value was calculated from four random field observations of two replicate experiments, and a minimum of 100 cells per field were counted.

2-2. Results

2-2-1. Isolation and purification of MC14

The active fractions collected from solvent extraction and open column chromatography were confirmed by thin layer chromatography with a R_f value of 0.7-0.75. In the final purification by the Inertsil SIL HPLC column, a single peak of

MC14 was observed and assayed the neurite outgrowth promoting activity as described in section 1-1-6. The HPLC profile of MC14 is shown in Fig. 2-1. The retention time of MC14 eluted with chloroform-methanol (98:2) with the flow rate of 0.5 ml/min was about 24 min. MC14 was then stored in methanol at 4°C for further bioassay and physicochemical characterization.

2-2-2. Appearance and solubility

MC14 showed pale yellow oil after freeze-dried. The purified MC14 was dissolved in methanol as a stock solution for several analyses. MC14 was soluble in toluene, diethyl ether, chloroform, acetone and methanol.

2-2-3. UV/VIS absorption spectrum of MC14

The absorption spectrum of MC14 showed that the maximum absorption peak was seen at 251 nm (Fig. 2-2).

2-2-4. Structure elucidation of MC14

The molecular structure of MC14 was elucidated by ^{1}H- and ^{13}C-NMR. The ^{13}C-NMR spectrum of MC14 showed 27 resolved peaks, which appeared to be 5 -CH_3, 7 -CH_2-, 6 =CH-, 6 =C<, 2 >C=O (ketone) and 1 -COOH- by analysis of the DEPT spectra. From the ^{13}C-NMR spectrum, the chemical shifts at 188.9 and 189.5 ppm suggested a keto-carbonyl moiety, which represents a typical benzoquinone structure. Since the ^{1}H-NMR spectrum displayed peaks at 6.566 and 6.433 ppm with low spin coupling constant, they represented the split protons. Based on COSY spectrum (data not shown),

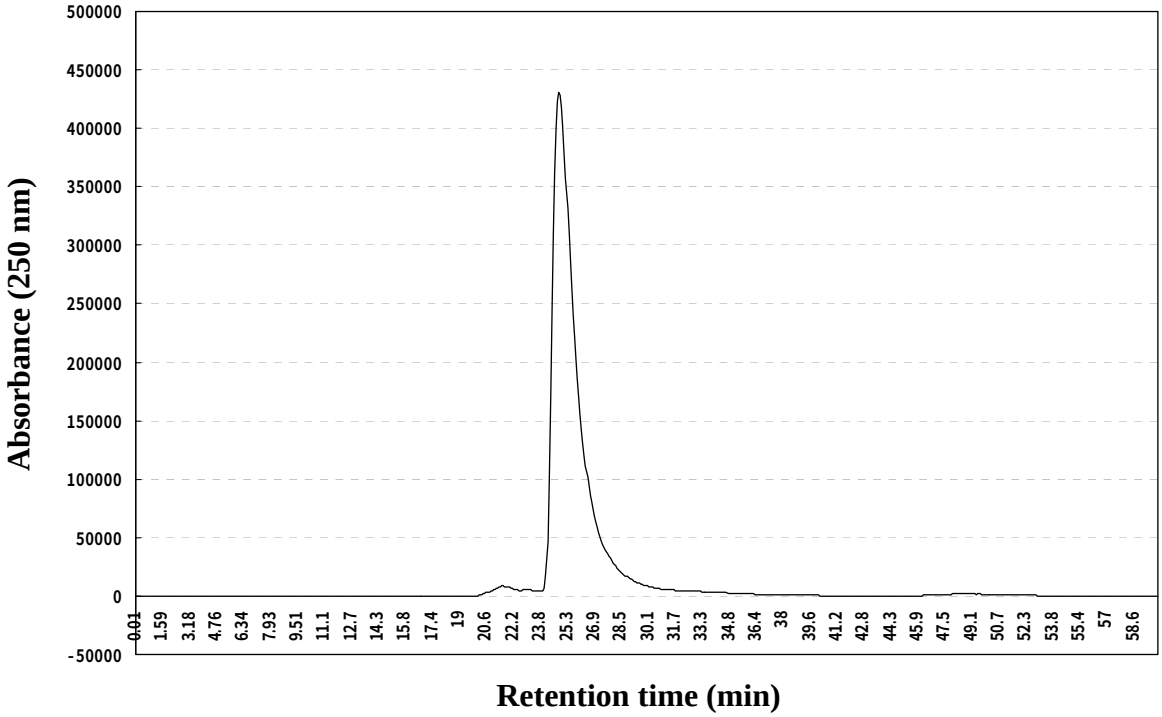


Fig. 2-1. HPLC profile of MC14 on Inertsil SIL column using $CHCl_3$-MeOH (98:2) as the eluting solvents at a flow rate of 0.5 ml/min and detected by the absorbance at 250 nm.

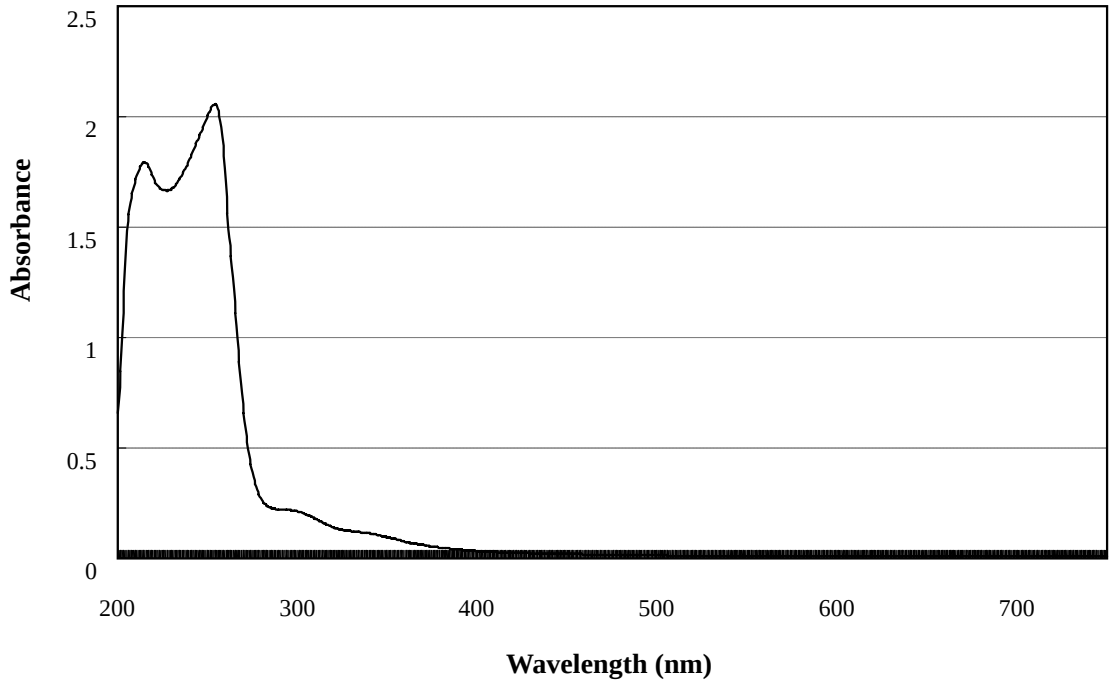


Fig. 2-2. UV/VIS absorption spectrum of MC14

the signal peaks at 6.566 ppm and 6.433 ppm were resulted from the long-range couplings from CH_3 (δ 2.027) and CH_2 (δ 3.123), respectively. These data suggested the partial structure as shown in Fig. 2-3. In addition, the HSQC spectra (data not shown) suggested the following partial structures in MC14:

=CH (5.808ppm)-CH_2 (2.502 ppm) -CH_2 (2.08 ppm)-

=CH (5.130 ppm)-CH_2 (2.14 ppm)-

=CH (5.087 ppm) – CH_2 (2.09 ppm) –

Besides, the main structure of MC14 was elucidated based on the analysis of HMBC spectra (data not shown). For the ease of identification, the C atoms in the chemical structure of MC14 is assigned with number (Fig. 2-4). The long range coupling from CH_3-proton (indicated by thick lines) is shown in Fig. 2-5A. In consistent with the 14' (CH) and 15' (C)-^{13}C, the chemical shifts of CH_3 at 1.575 and 1.661 ppm were assigned to the position at 16' and 20' based on the HMBC spectra (the difference between 16' and 20' was determined by NOE spectra, data not shown). The long-range couplings from proton at 1' are shown in Fig. 2-5B (think lines), Together with the interpretation of Fig. 2-5A, the partial structure from 1' to 4' ,and 17', as well as a quinone structure were determined. On the other hands, the chemical shift of COOH (δ 172.0) and the protons between CH_2 (δ 2.21) to CH (δ 5.808) were observed in HMBC spectra. The couplings from protons in –CH= (δ 5.808) are shown in Fig. 2-5C (think lines) suggested that a COOH group is linked to position 6'-12'. The carbon chain of MC14 was confirmed by the peaks appeared between the 5'-4' and 6'-5' in HMBC, H-12'(δ 2.21) and the 13',14'-^{13}C peaks in the HMBC spectra. Besides, the structure in the Fig.2-5D (thick lines) was elucidated by TOCSY spectra (data not shown). According to NOESY spectra, the NOE between =CH- and CH3 was only observed at the H-14' (δ

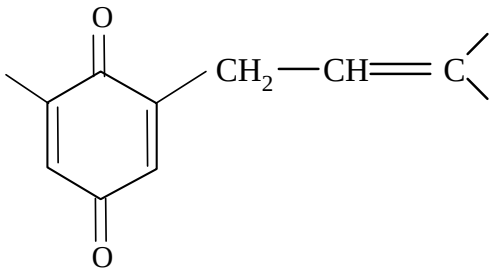


Fig. 2-3. Partial structure of MC14 determined by NMR

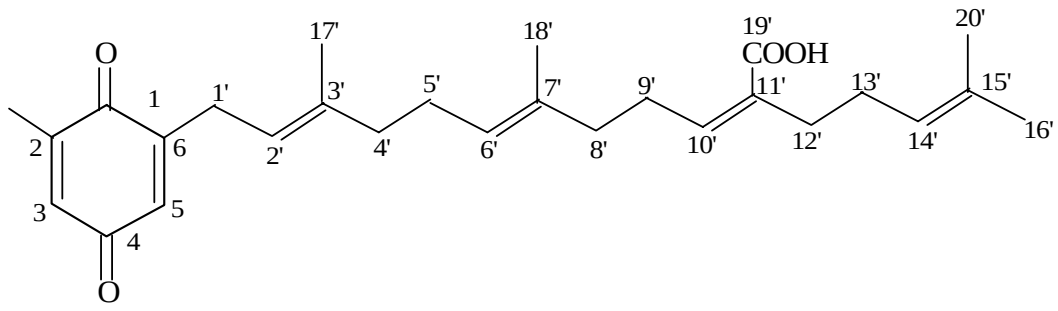


Fig. 2-4. Chemical structure of MC14 determined by NMR analysis.

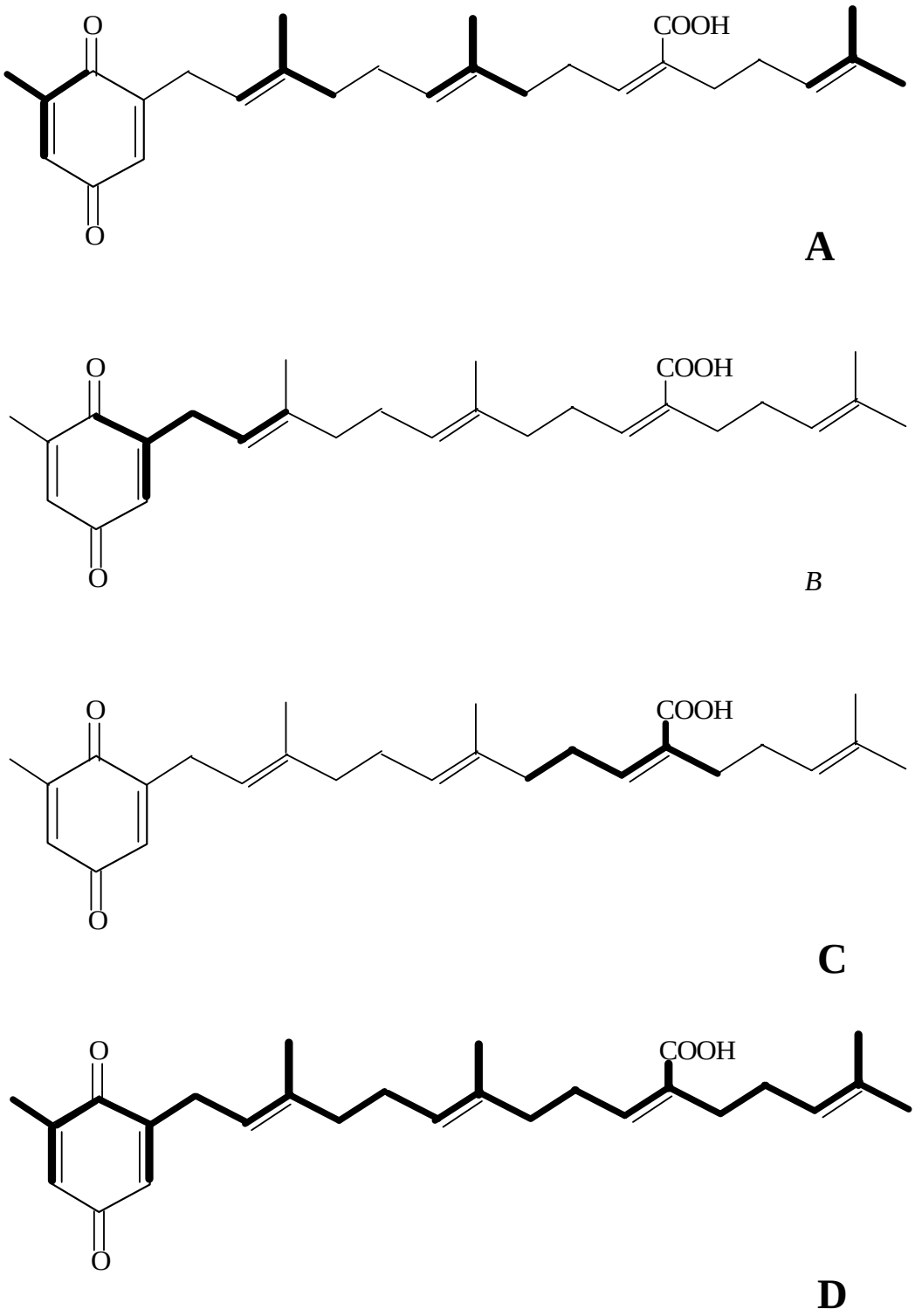


Fig. 2-5. The main chemical structure determined by HMBC spectra (A-C) and TOCSY spectra (D).

5.087) and the signal peak at 1.661 ppm, indicating that the chemical shift at 1.661 ppm represented H-16'. NOE of (2',4') and (10',12') indicates the presence of trans-double bonds at 2' and 10'. Although the NOE of the H-6'was overlapped with the protons at 4', 8' and 13' and caused it difficult to be observed, its broad cross peak suggested that it should be appeared at 4' and 8' position. Since NOE was not observed at 6' and 18', the double bond at 6' was also a trans conjugated substitution. The assignments of NMR are summarized in Table 2-1.

Together with the data from high resolution EI-MS, which showed an ion peak at m/z = 424, the molecular formula of MC14 was elucidated to be $C_{27}H_{36}O_4$ with molecular mass of 424 and its chemical structure is shown in Fig. 2-6.

2-2-5. Neurite outgrowth promoting activity of MC14

MC14 significantly enhanced the proportion of cells with neurite in the presence of 10 ng/ml NGF in dose dependent manner (Fig. 2-6). At 6.25 μg/ml MC14, approximately 5-fold and 1.5-fold increases in the proportion of neurite-bearing cells were noted compared with that of negative control and positive control, respectively. Optimal effective concentration of MC14 was observed at 6.25μg/ml. Cytotoxic effect on PC12D cells was observed at 12.5 μg/ml MC14.

2-3. Discussion

The neurite outgrowth promoting substance, MC14 was extracted from the brown alga *Sargassum macrocarpum* with methanol and chloroform, and successfully purified by silica gel column chromatography, size exclusion chromatography and finally two

Table 2-1. ^{1}H and ^{13}C-NMR chemical shifts of MC14.

Carbon number	^{13}C chemical shifts (ppm)	^{1}H chemical shift (ppm)	J value (Hz)
1	189.5	---	
2	147.6	---	
3	134.0	6.566	
4	188.9	---	
5	133.1	6.433	$J_{5,1'}$=1.6
6	150.0	---	
2-CH3	*	2.027	$J_{CH3,3'}$=1.5
1’	28.6	3.123	$J_{1',2'}$=7.3
2’	120.1	5.179	
3’	140.5	---	
4’	40.7	2.09	
5’	27.3	2.14	
6’	125.7	5.130	$J_{5',6'}$=7.0
7’	135.9	---	
8’	40.3	2.08	
9’	29.1	2.502	$J_{9',8'}$=7.3
10’	141.9	5.808	$J_{9',10'}$=7.3
11’	133.9	---	
12’	36.1	2.21	
13’	29.0	2.09	
14’	124.8	5.087	$J_{13',14'}$=7.0
15’	132.9	---	
16’	25.9	1.661	
17’	*	1.649	
18’	16.0	1.600	
19’	172.0	---	
20’	17.8	1.575	
	*16.1, 16.2		

Chemical shifts are shown with reference to CD_3OD.

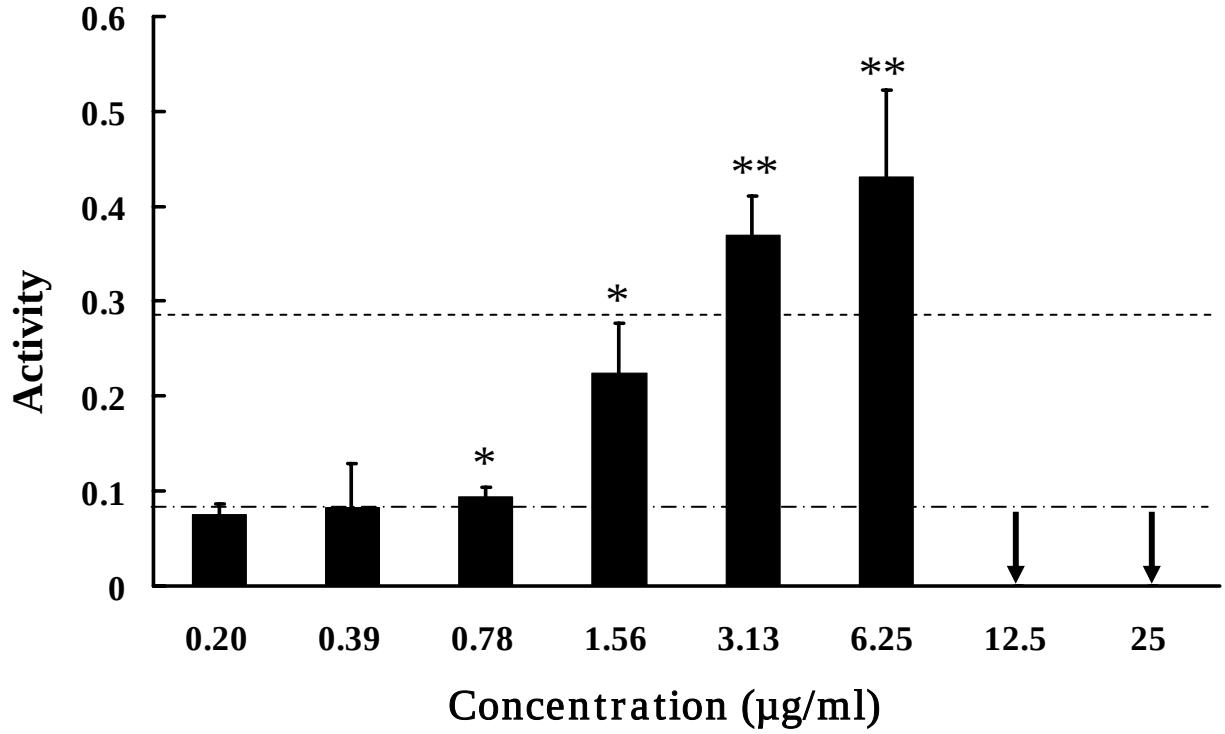


Fig. 2-6. Neurite outgrowth promoting activity of MC14. PC12D cells treated with the indicated concentration of MC14 in the presence of 10 ng/ml NGF for 48 h. Cells treated with 50 ng/ml NGF (------) and 10 ng/ml (−·−·−) were used as positive and negative controls, respectively. Arrows indicate cytotoxicity. Significant difference from the negative control: ****p* < 0.05; ** *p* < 0.01 (Student's *t*-test).

steps of HPLC. Approximately 3.4 mg of the MC14 was purified from 500 g wet weight of the algal sample. Compared with the previous extraction procedure, much lesser amount of chloroform was used and therefore the cost for the initial solvent extraction can be reduced. Besides, the handling of methanol is safer than chloroform in terms of the inhalation of the toxic and volatile chloroform vapor.

Based on the NMR analyses, the structure of MC14 was elucidated to be sargaquinoic acid, which is recognized to have a novel neurite outgrowth promoting activity. Sargaquinoic acid was first discovered and purified by Kusumi *et al* (1979) from the methanol extract of a brown alga *Sargassum serratifolium* during a course of the investigations on the constituents of algae. It also has been reported that sargaquinoic acid is found in a brown alga *Sargassum sagamianum* (Segawa & Shirahama, 1987). These findings suggest that this naturally occurring quinone may be a unique metabolites in the family of *Sargassaceae*. Surprisingly, during a screening program conducted in our laboratory, we found that only the extract from *Sargassum macrocarpum* showed potent neurite outgrowth promoting activity out of 30 *Sargassum* species (Table 2-2). This result suggests that *S. macrocarpum* contains a particularly high level of sargaquinoic acid, implying that this species of brown alga will be a rich source of this useful compound. On the other hand, the screening of more *Sargassum* species will be important for identifying the other natural sources of MC14.

The elucidation of the chemical structure of MC14 will be very crucial for further analysis of its structure-activity relationship, which will be discussed in chapter VIII, as well as the synthesis of this useful chemical in the chemical industry.

Table 2-2. *Sargassaceae* family tested in the screening program of marine algae collected from Japan coastline

Species	Japanese name
Hizikia fusiformis 1	Hijiki 1
Hizikia fusiformis 2	Hijiki 2
Sargassum -	Ezonnejimoku
Sargassum -	Hahakimoku
Sargassum alternato-pinnatum	Kirebamoku
Sargassum confusum	Hushisujimoku
Sargassum crassifolium	Atsubamoku
Sargassum crispifolium	Kobukuromoku
Sargassum cristaefolium	Tosakamoku
Sargassum duplicatum	Hutamoku
Sargassum filicinum	Shidamoku
Sargassum fulvellum	Hondawara
Sargassum hemiphyllum	Isomoku
Sargassum horneri	Akamoku
Sargassum ilicifolium	-
Sargassum macrocarpum	Nokogirimoku
Sargassum micracanthum	Togemoku
Sargassum miyabei	Miyabemoku
Sargassum nipponicum	Tamanashimoku
Sargassum oligocystum	-
Sargassum patens	Yatsumatamoku
Sargassum piluliferum	Memetawara
Sargassum polycystum	Kobamoku
Sargassum polyporum	-
Sargassum ringgoldianum	Oobamoku
Sargassum ringgoldianum	Yanagimoku
Sargassum siliquastrum	Toremoku
Sargassum sp. 1	Hondawara sp. 1
Sargassum sp. 2	Hondawara sp. 2
Sargassum thunbergii	Uminotoranoo
Sargassum yendoi	Endoumoku
Turbinaria ornata	Rappamoku

Chapter III

Distribution of MC14 in *Sargassum macrocarpum*

In chapter II, the neurite outgrowth promoting substance, MC14 was found in the brown alga, *Sargassum macrocarpum*, and also successfully isolated and determined its chemical structure. At the early stages of the extraction from *Sargassum macrocarpum*, one of the most obvious questions to ask is whether the natural product of interest is localized in the same particular portion of the organisms. In the case of marine algae, it is fairly straightforward to determine the localization of the compounds of interest as the morphological structure of most marine algae can be divided into leaves, root, stem or air-sac for some species. If so, this may allow for disposal of the certain part of algal plant before the actual extraction work begins, thus rendering the crude extract less complex and possibly leading to avoidance of problems brought about by the presence of other parts of the algae. Besdies, less extraction solvent and higher yield of purification can be achieved. Accordingly, the distribution of MC14 in *S. macrocarpum* should be proved for further efficient extraction of MC14. Thus, the distribution of MC14 in *S. macrocarpum* was studied and is described in this chapter.

3-1. Materials and Methods

3-1-1. Preparation of crude extracts

After washing with ASW and PBS as described in detailed in chapter II, the plant

body of *S. macrocarpum* was divided into leaf, stem and air-sac based on its morphological structure. Each portion (50 g) was homogenized in 200 ml PBS with a domestic mixer. After centrifugation at 5000g for 20 min, the supernatant (PBS extract) was collected and used for bioassay. The residue was extracted with 200 ml methanol by a mechanical homogenizer (Polytron) at 1,3000 rpm and the methanol-extract was collected after filtration, evaporated to complete dryness for dry weight determination. The tested concentration was prepared by dissolving the extract in suitable amount of methanol. The test concentration of PBS-extract was prepared by diluting the original solution with PBS.

3-1-2. Bioassay of neurite outgrowth promoting activity

The bioassay test was conducted as described in section 2-1-8.

3-2. Results and Discussion

As shown in Fig. 3-1, the morphology of the brown alga *S. macrocarpum* is mainly composed of stem, leaf and air-vesicle. They are divided from the plant body and extracted separately for the bioassay of neurite outgrowth promoting activity. The results showed that the chloroform-extract of stem (10 μg/ml) could markedly enhance the neurite outgrowth from PC12D cells in the presence of 10 ng/ml NGF compared with negative control. By contrast, no significant activity was observed for the chloroform-extract of leaf or air-vesicle even at 100 μg/ml (Fig. 3-2A). Regarding the PBS-extract, none of the algal portion showed significant neurite outgrowth promoting activity (Fig. 3-2B).

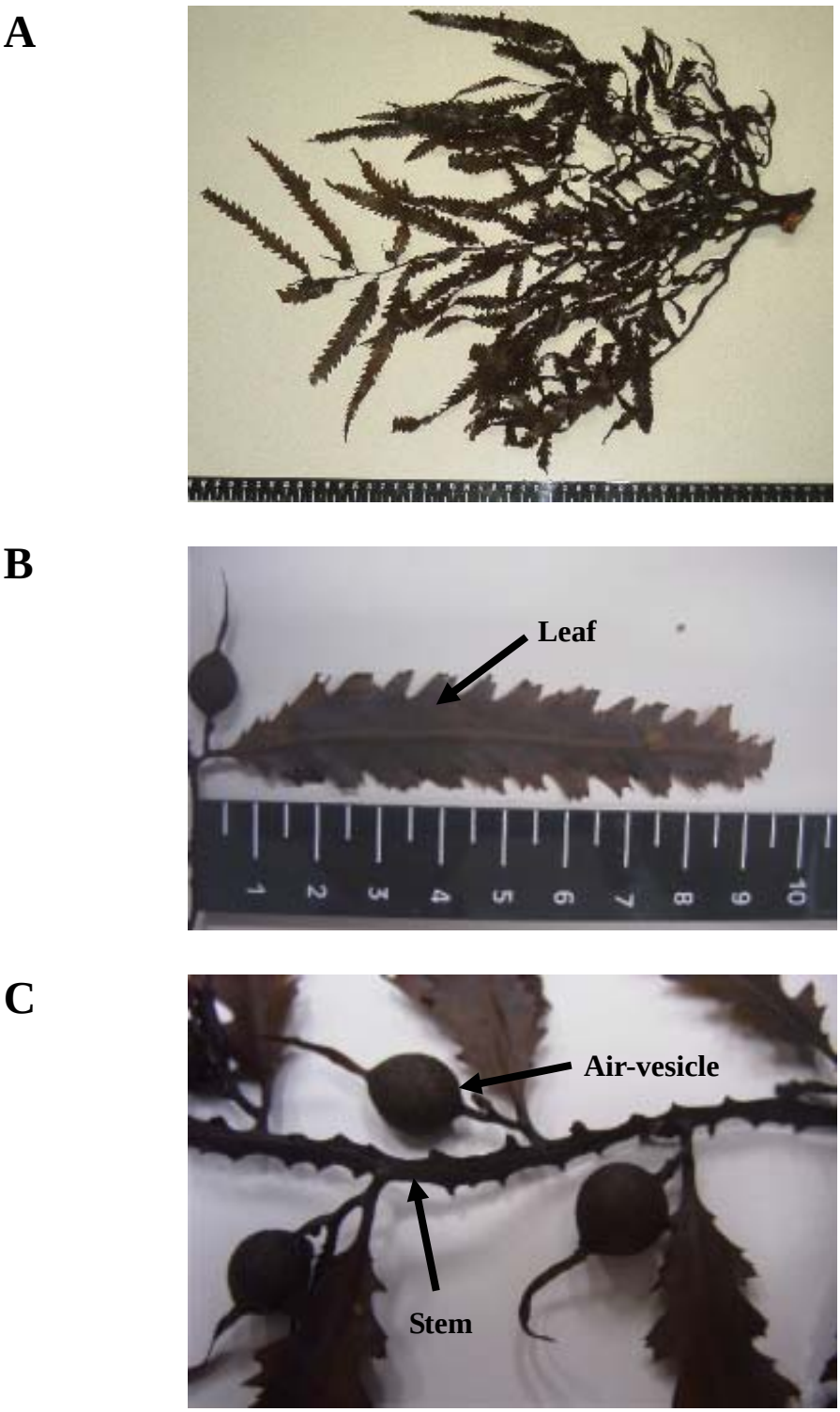


Fig. 3-1. (A) Morphology of *S. macrocarpum*. (B) An enlarged photo showing a leaf of the alga. (C) The air-vesicle is a spherical hollow structure branched out from the stem region of the alga.

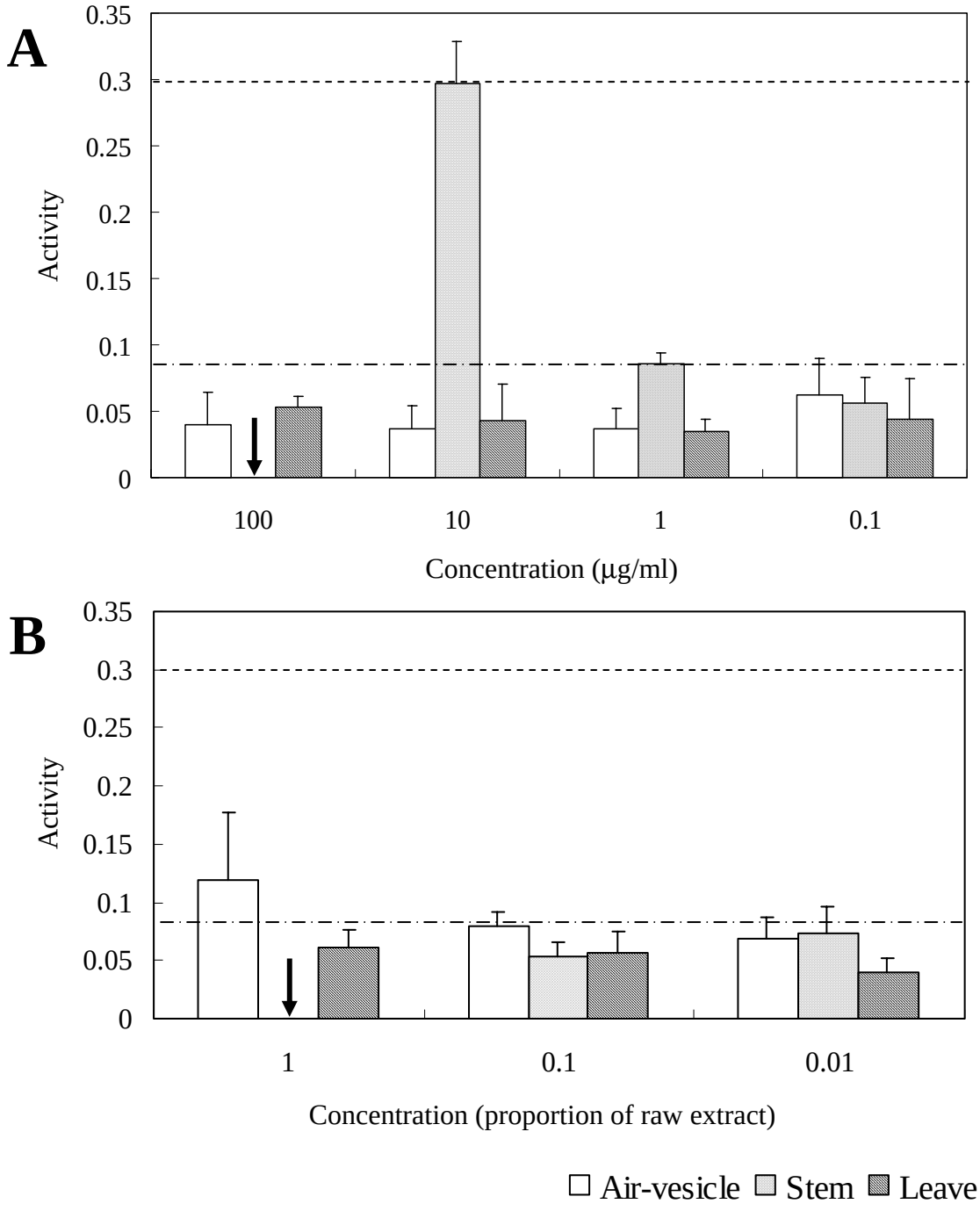


Fig. 3-2. Neurite outgrowth promoting activity of chloroform- and PBS-extract from stem, leave and air-vesicle of *S. macrocarpum*. PC12D cells were treated with the indicated concentrations of (A) chloroform-extract and (B) PBS-extract in the presence of 10 ng/ml NGF for 48h. Cells treated with 50 ng/ml (--------) and 10 ng/ml NGF (_ . _ . _) were used as positive and negative controls, respectively. Arrows indicate cytotoxicity.

These findings clearly show that the stem region of *S. macrocarpum* contains high level of the neurite outgrowth promoting substance while the air-vesicle or leaf may not contain any neurite outgrowth promoting substance or its level is too low to be detected. Hornsey and Hide (1976a; 1976b) have examined 151 species of British algae and determined species activity, seasonal variation, and selective concentration within the algal thallus. Although the reason underlying the uneven distribution of this active compounds in *S. macrocarpum* is not known, the important finding of this study is that during the purification of MC14 from this alga, it is possible to dispose almost half of the plant by weight before the initial solvent extraction and concentrate on the extraction of the stem portion. Therefore, direct extraction from this algal stem is likely to make easier the extraction and purification of MC14. More importantly, smaller volume of organic solvent can be used and the yield would also be elevated compared with the extraction from the whole bulk of biomass.

Chapter IV

Neurite Outgrowth Promoting Activity of MC14

Nerve growth factor (NGF) are essential for the normal development and functional maintenance of nerve cells in both central nervous system and peripheral nervous system. The most classical and representative effect of NGF is its ability to induce the outgrowth of neurite from sensory, sympathetic and certain central cholinergic neurons to their targets during developmental stage of the nervous system (Matthews, 1998). NGF has been demonstrated to play a crucial role in regulating the distribution of axon processes within the target tissues to establish functional synaptic connections with them (Campenot, 1977).

The rat pheochromocytoma PC12 cell line is widely used as a neuronal cell model system for the investigation of neurotrophic action of NGF because they respond to NGF by extending neurite and differentiating into sympathetic neuron-like phenotype (Greene & Tischler, 1976). Recently, a subline of PC12 cells (PC12D cells) has been established. PC12D cells were found to be capable of responding to NGF in a faster rate by extending neurites within 48 h (Katoh-Semba *et al.*, 1987). This property of PC12D cells made them an ideal cell model system for screening the neuroactive substances as well as studying the biological effect of NGF and NGF-potentiating substances. As a first step to explore the NGF-potentiating activity of the purified active substance MC14, the NGF-induced neurite outgrowth promoting activity of MC14 to PC12D cells, as well as the neurotrophic effect of MC14 in the absence of NGF on PC12D cells were evaluated. The concentration-dependent effect of MC14 and its promoting activity in

the presence of different concentrations of NGF were also analysed. In addition to the study of neurite outgrowth promoting effect of MC14 during the initial differentiating stage of PC12D cells, the chronic neurite outgrowth maintaining effect of MC14 was also investigated in order to pursue a more comprehensive study on the neuritogenic action of MC14.

In the second part of this chapter, the signaling cascades mediating the NGF-induced and MC14-enhanced neurite outgrowth in PC12D cells will be described in an attempt to elucidate the mechanisms of action of MC14 in PC12D cells. Cumulative efforts from many laboratories over the past decade have led to the discovery and synthesis of various specific inhibitors specific to the signaling molecular and effector proteins involved in intracellular signal transduction pathways. The utilization of these signaling molecule inhibitors has provided a powerful tool and pharmacological evidence for the elucidation of how the complicated intracellular signaling cascades mediate the signals initiated by the nerve growth factor leading to its great diversity of cellular processes such as proliferation, differentiation, development and apoptosis of the neuronal cells. In the present study, the signaling pathways that mediate the NGF-induced neurite outgrowth from PC12D cells, and the mechanisms of action of MC14 on promoting NGF-induced neurite outgrowth were elucidated using several representative signaling molecule inhibitors which have already been known to specifically inhibit certain signaling molecules or effector proteins in the signaling cascades.

Since most of the NGF-induced cellular responses are initiated by the binding of NGF to its specific tyrosine receptor kinase type A (TrkA), and subsequently activating the downstream protein kinases and effector proteins (Kaplan *et al.*, 1991a; Kaplan *et al.*, 1991b), the effect of MC14 on binding of NGF to the surface receptor TrkA was

investigated as a first step to investigate its mechanisms. To achieve this objective, a general protein kinase inhibitor K252a was used because it can act as potent inhibitor of TrkA receptor at 3 nM while it can inhibit many other protein kinase including PKA and PKC at a concentration higher than 25 nM (Hashimoto, 1988; Koizuma *et al*., 1988; Tapley *et al*., 1992; Pawlowaka *et al*., 1993). In addition, a high dose of K252a was tested to confirm the role of potential protein kinases on the action of MC14 in PC12D cells. Concerning the involvement of MAPK-mediated signaling pathway in the MC14-stimulated neurite outgrowth enhancement on PC12D cells, a specific mitogen-activated protein kinase (MAPK) kinase inhibitor PD98059 was used in the study. Mitogen-activated protein kinases are a group of protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli (e.g. NGF) and mediate signal transduction from the cell surface to the nucleus. The selective inhibitor of MAP kinases PD98059 acts by inhibiting MAP kinases and subsequent phosphorylation of MAP kinase substrates. It has been reported that the pretreatment of the traditional PC12 cells with PD98059 completely blocked the 4-fold increase in MAP kinase activity produced by NGF at 2 μM. However, it has no effect on NGF-dependent tyrosine phosphorylation of the TrkA receptor or its substrate Sch and did not block NGF-dependent activation of PI 3-kinase (Dudley *et al*., 1995; Pang *et al*., 1995; Waters *et al*., 1995). Hence, PD98059 is an ideal inhibitor on studying the role of MAPK kinase-mediated signal pathway for MC14 activity to PC12D cells. With respect to the involvement of PKA-mediated signaling pathway in response to MC14 on PC12D cells, a PKA inhibitor was used as it can effectively block the signal transduction of cyclic adenosine monophosphate (cAMP) by inhibiting PKA at K_i of 2-8 nM (Santa cruz biotechnology, Inc.). Finally, another potent and specific inhibitor of protein kinase C

(PKC, IC_{50} = 0.66 μM), chelerythrine chloride, was also selected to determine the involvement of PKC for MC14-stimulated activity because it can interact with the catalytic domain of PKC and may also affect the translocation of PKC from cytosol to plasma membrane (Herbert *et al*., 1990; Eckly-Michel *et al*., 1997; Chao *et al*., 1998;). These studies contributed to the elucidation of the mechanism of action of MC14 on neurite outgrowth promoting activity to PC12D cells.

4-1. Methods and Materials

4-1-1. Medium and reagents

The following chemicals were obtained from the indicated sources: Dulbecco's modified Eagle's medium (DMEM, high glucose) and horse serum (HS) from Gibco RBL; fetal bovine serum (FBS), 2.5 S NGF (mouse submaxillary glands), poly-L-lysine (MW 30,000-70,000), penicillin, streptomycin, ampicillin and chelerythrine chloride from Sigma. K252a and PD98059 from Calbiochem; protein kinase A (PKA) inhibitor from Santa Cruz Biotechnology.

4-1-2. Cell culture

Rat pheochromacytoma PC12D cells were obtained from Dr. M. Sano (Aichi Human Service Center, Japan). Cultures of PC12D cells were maintained in DMEM supplemented with 10% HS, 5% FBS, 100 U/ml penicillin, 200 μg/ml streptomycin and 25 μg/ml ampicillin (complete medium) in a water-saturated atmosphere of 5% CO_2 at 37°C.

4-1-3. Bioassay of neurite outgrowth promoting activity of MC14

PC12D cells were harvested and seeded at a cell density of 5 X 10^3 cells per well on 96-well tissue culture plate coated with poly-L-lysine (10 μg/ml). After 24 h incubation in humidified air (5% CO_2) at 37° C in complete medium, MC14 (3 μg/ml) and NGF (0-100 ng/ml) were added to the cells. In the experiment, MC14 was dissolved in MeOH and added to the cells in less than 1% v/v of medium, which was determined to have no effect on PC12D cells. After 48 h incubation, the proportion of neurite-bearing cells were determined with a microscope with 200X magnification. Process with length longer than two diameters of the respective cell body was counted as neurite. For each datum point, the mean value was calculated from four to six random field observations of two replicate experiments, and a minimum of 100 cells per field were counted.

4-1-4. Dose response assay of MC14

PC12D cells were harvested and seeded on the tissue culture plate as described in section 4-1-1. After 24 h incubation in complete medium, MC14 was added at a concentration range from 0.2 to 6.25 μg/ml (predetermined highest concentration of MC14 without causing cytotoxic effect on PC12D cells) in the presence of 10 ng/ml NGF (the predetermined optimal concentration of NGF for observing neurite outgrowth promoting activity of MC14). Cells were treated with 10 ng/ml and 50 ng/ml NGF alone for comparing the effect of MC14. After 48 h incubation, the proportion of neurite-bearing cells were determined as described above.

4-1-5. Assay of chronic neurite outgrowth supporting effect of MC14

PC12D cells were seeded on the tissue culture plate as described above. After 24 h

incubation, the medium was changed to fresh medium containing 6.3 μg/ml MC14 and 1.25-25 ng/ml NGF. Medium in each well was renewed every 3 days to maintain a constant concentration of MC14 and NGF throughout the experimental period. At the same time, effect of NGF alone was also tested by adding NGF into the medium only. Neurite outgrowth of PC12D cells was monitored regularly for an experimental period of 28 days using a phase-contrast microscope.

4-1-6. Inhibitors to MC14-stimulated neurite outgrowth

PC12D cells were seeded on the tissue culture plate and incubated for 24 h as described above. Medium was changed with fresh complete medium containing the tested inhibitor and incubated for 1 h before the additions of MC14 and NGF. After 48 h incubation, the proportion of neurite bearing cells were determined as described above. The following stock concentrations of the inhibitors dissolved in the indicated solvent were used in the experiments: PD98059 (20 mM, DMSO); PKA inhibitor (5 mg/ml, DMEM); chelerythrine chloride (0.5 mg/ml, DMEM); K252a (200 μM, MeOH). Each inhibitor was further diluted with DMEM to a suitable working concentration in the experiment and aliquot of the working concentration of each inhibitor was added to the test well in less than 1% v/v of medium, in which no solvent effect was observed on PC12D cells. Because the absolute number of neurite-bearing cells in NGF-treated cultures varied slightly between experiments, the effects of inhibitor were compared with those of NGF treatment on positive control culture (50 ng/ml NGF) from the same experiment.

4-1-7. Morphological observation of PC12D cells

The morphological changes of PC12D cells after various treatments were recorded using a phase-contrast microscope (Olympus IMT2) with 200-400X magnification. Photo-slides were taken using a compatible Olympus SC-35 type camera which connected serially to the phase-contrast microscope. Image was scanned from the slide and transformed into computer file using Polaroid Polascan 35/LE system. Contrast of the image was adjusted if necessary.

4-1-8. Statistical analysis

Each datum point is the mean ± S.D. of four-six random field observations from at least two replicate experiments. Significant difference from the control was determined by the Student's *t*-test. $P < 0.05$ was considered to be a significant difference.

4-2. Results

4-2-1. Morphological changes of PC12D cells treated with NGF and MC14

When cultured in complete medium only, PC12D cells were in round shape without neurite extension (Fig. 4-1A). In the presence of 10 ng/ml NGF, only a few neurite-bearing cells were observed (Fig. 4-1B) while 50 ng/ml NGF caused a robust outgrowth of neurite from PC12D cells after 48 h (Fig. 4-1C). Treatment of cells with 6.3 μg/ml MC14 and 10 ng/ml NGF markedly enhanced the number of neurite-bearing cells. The growth cone was observed at the tip of extending neurite, as those induced by 50 ng/ml NGF (Fig. 4-1D). Besides, the average neurite length of the MC14-treated cells was comparable to those induced by 50 ng/ml NGF.

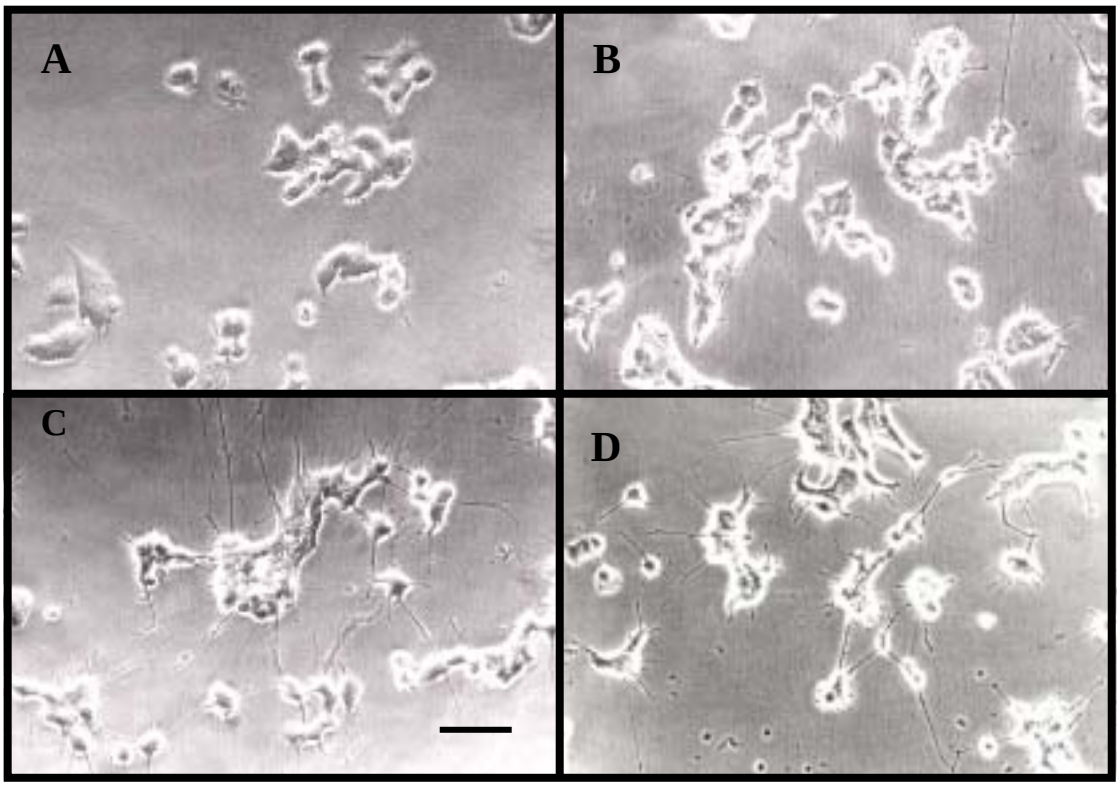


Fig. 4-1. Phase-contrast micrographs of PC12D cells after various treatments for 48 h. (A) Untreated control culture. (B) PC12D cells treated with 10 ng/ml NGF only. (C) PC12D cells treated with 50 ng/ml NGF only. (D) PC12D cells treated with 6.3 μg/ml MC14 in the presence of 10 ng/ml NGF. Scale bar: 20 μm.

4-2-2. Effect of NGF on neurite outgrowth promoting activity of MC14

Addition of 50 ng/ml NGF induced 25-31% of PC12D cells to extend neurite after 48 h (Fig. 4-2). The proportion of neurite-bearing cells did not significantly increase at 100 ng/ml NGF treatment, suggesting that 50 ng/ml NGF was the saturating concentration for neurite outgrowth of PC12D. Addition of MC14 (3 μg/ml) together with the saturating concentrations of NGF (50 ng/ml and 100 ng/ml) further enhanced the proportion of cells with neurite up to a 2-fold enhancement compared with the cultures treated with NGF alone. Besides, significant neurite outgrowth enhancing activity of MC14 was observed in the presence of relatively low concentration of NGF ranging from 1.25-25 ng/ml ($P < 0.01$, Student's *t*-test). A maximum of approximately 50% neurite-bearing cells was recorded at cultures treated with 100 ng/ml NGF in the presence of MC14. Although MC14 exhibited strong activity in enhancing NGF-induced neurite outgrowth, MC14 alone could not show any neurotrophic action on PC12D cells. It can be observed that the addition of MC14 to PC12D cells shifted the dose-response curve of NGF to the left (Fig. 4-2), implying that in the presence of MC14, either a greater number of NGF receptors were activated so that the NGF-receptor mediated intracellular signaling pathway was amplified, or that MC14 and NGF might employ distinct but complementary signal transduction cascades.

4-2-3. Dose-dependent activity of MC14

The action of MC14 was dose-dependent (Fig. 4-3). The minimal effective concentration of MC14 was about 0.8 μg/ml in the presence of 10 ng/ml NGF. A linear neurite outgrowth enhancing effect of MC14 was observed from 0.8-6.3 μg/ml. At 6.3 μg/ml MC14, a 5-fold enhancement of the number of neurite-bearing cells was recorded

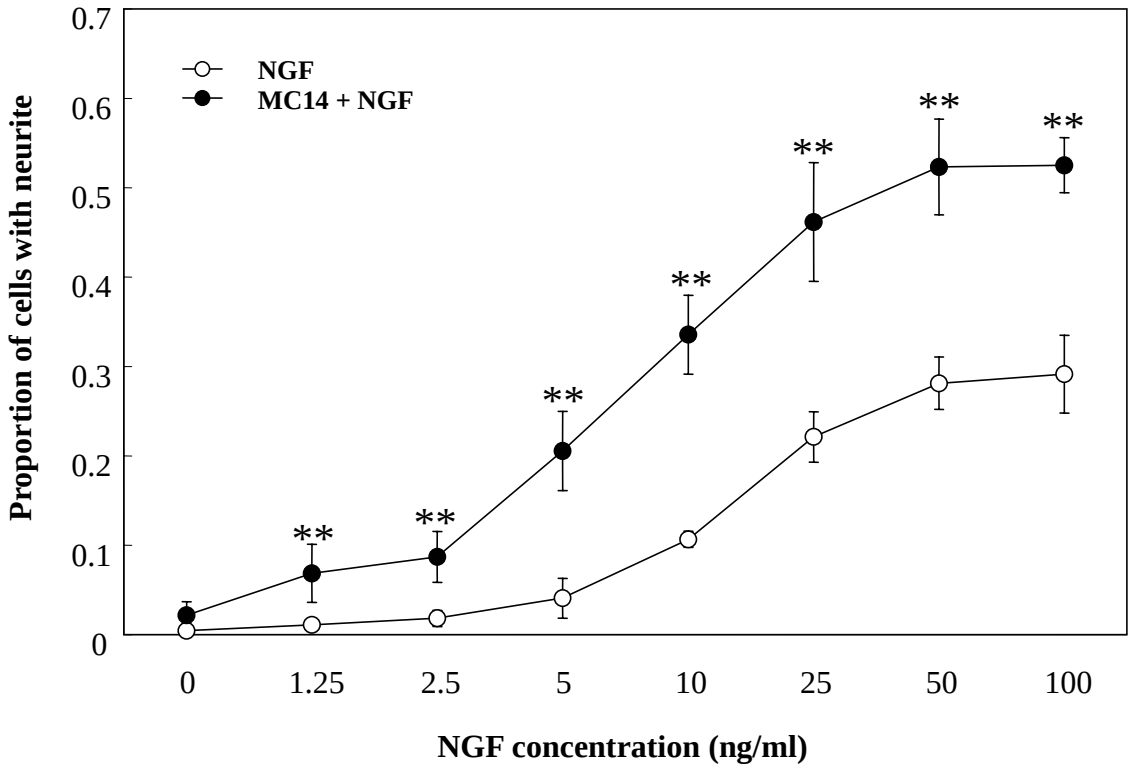


Fig. 4-2. Neurite outgrowth promoting activity of MC14 in the presence of NGF. PC12D cells were treated with or without 3 μg/ml MC14 in NGF for 48 h. Each point represents the mean ± SD from two replicate experiments. Significant difference from the NGF-only control: ** *P* < 0.01 (Student's *t*-test)

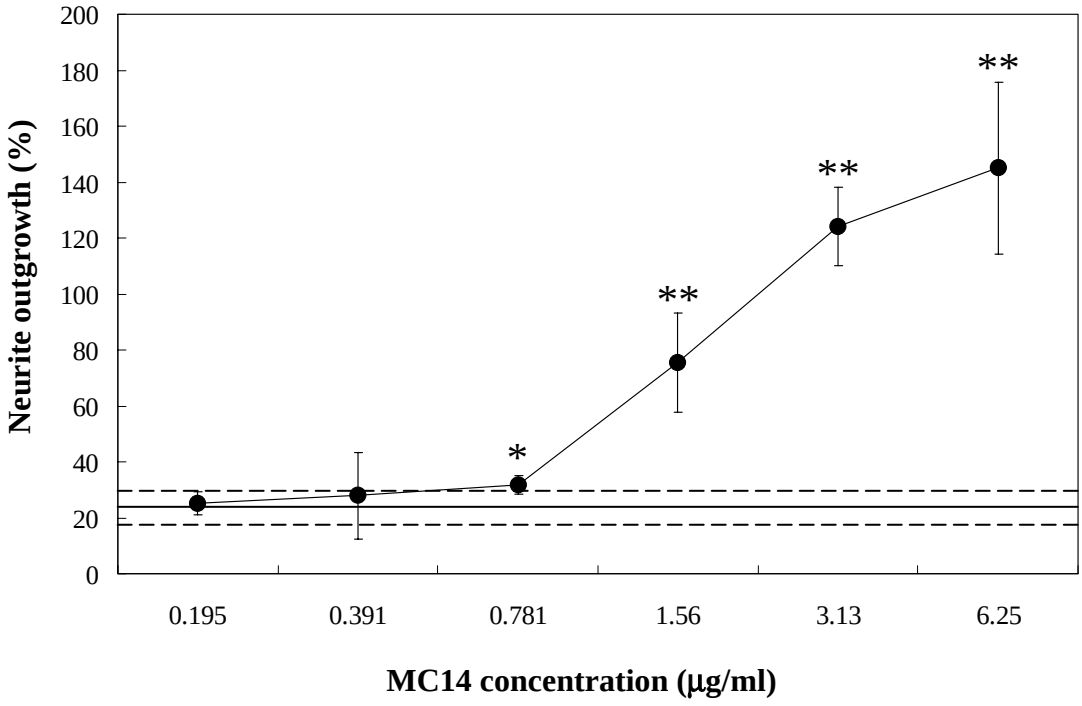


Fig. 4-3. Dose-response curve of the neurite outgrowth enhancing effect of MC14 on PC12D cells in the presence of 10 ng/ml NGF. The horizontal solid line represents the mean value for cultures treated with 10 ng/ml NGF alone, the dotted lines represent the SD of this value. Neurite outgrowth is expressed as a percentage relative to the saturated concentration of NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the NGF-only control: * *P* < 0.05; ** *P* < 0.01 (Student's *t*-test).

compared to the cultures treated with 10 ng/ml and 50 ng/ml NGF alone, respectively.

4-2-4. Chronic neurite outgrowth enhancing effect of MC14 on PC12D cells

As MC14 showed an acute effect on NGF-mediated neurite outgrowth enhancement on PC12D cells after 48 h, it is important to further investigate whether MC14 also exhibits a chronic effect on PC12D cells in terms of promoting and supporting the extended neurites from the cells. The chronic effect of MC14 on PC12D cells was studied by monitoring the morphology of MC14-treated PC12D cells for a period of 28 days with constant renewal of NGF and MC14 in the medium. The result showed that MC14 firstly promoted neurite outgrowth and subsequently maintained the outgrowth processes from PC12D cells. The morphological changes of PC12D after the long-term treatment with MC14 in the presence of NGF is shown in Fig. 4-4. Cells treated with 6.3 μg/ml MC14 in the presence of 50 ng/ml NGF extended short neurites after 24 h (Fig. 4-4B) and the neurite length further increased with incubation time (Fig. 4-4C,D). At day 7, some neurites had contacted to the neighboring cells and seemed to form a connection with those cells (Fig. 4-4E). Both the proportion of neurite-bearing cells and the observed average neurite length increased with the culturing time and the most striking observation was that a network-like neuritic connection among cells was constructed at day 14 (Fig. 4-5F). In contrast, much lower degree of network-like formation from the neurite-bearing cells was observed for the cells incubated in the same concentration of NGF alone because the cells only extended short neurites that failed to make contact with the nearby cells (Photo not shown). These observations suggest that MC14 does not only promote neurite outgrowth as an acute effect on PC12D cells, but also maintains the outgrowth of neurite and further enhances the

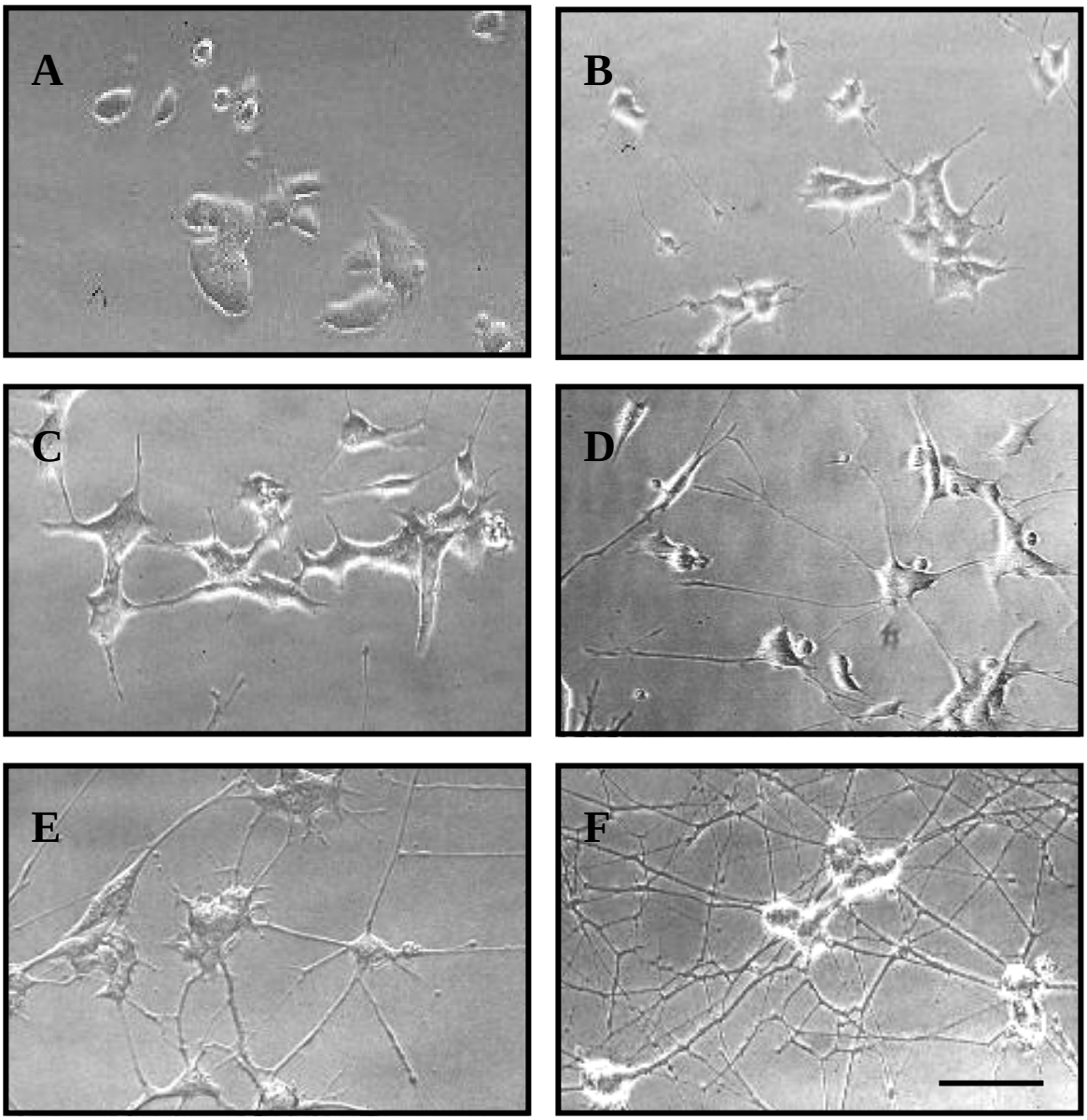


Fig. 4-4. Morphology of PC12D cells treated with 6.3 μg/ml MC14 and 50 ng/ml NGF for (A) 0, (B) 1 ,(C) 2, (D) 5, (E) 7 and (F) 14 days. The medium was renewed every 3 days to maintain the concentration of MC14 and NGF during the experiment. Scale bar = 20 μm

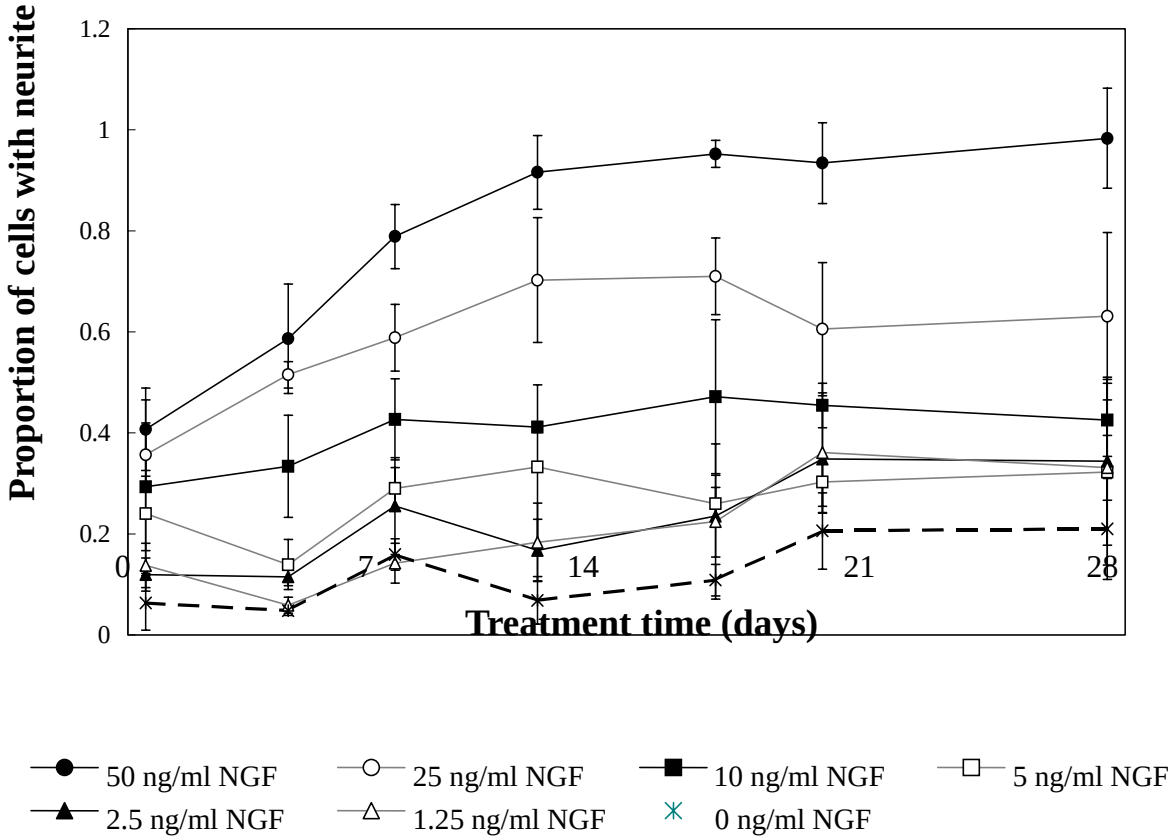


Fig.4-5. Long-term effect of MC14 on neurite outgrowth promoting activity on PC12D cells. Cells were treated with 6.3 μg/ml MC14 in the presence of NGF during the incubation period of 28 days. The medium was renewed every 3 days to maintain the concentration of MC14 and NGF during the experiment. Each datum point represents the mean ± SD (n=6).

extension of neurites for the ultimate formation of network-like structure among PC12D cells. The proportion of neurite-bearing cells for the cultures treated with 6.3 μg/ml MC14 in the presence of NGF at 1.25-50 ng/ml NGF is shown in Fig. 4-5. The treatment with MC14 in the presence of 50 ng/ml NGF resulted in an elevation of neurite-bearing cells from 40% at day 1 to 90% at day 12, and almost 100% of cells were bearing neurite at day 28, while only 40% of neurite-bearing cells was observed in the culture incubated with 50 ng/ml NGF (Fig.4-6). The similar pattern of neurite outgrowth, although in lesser extent, was observed for the MC14-treated cells in the presence of 25 ng/ml NGF. The percentage of neurite-bearing cells maintained at approximately 60% from day 12 to day 28, at which a 3-fold increase of the neurite-bearing cells was observed compared with the culture treated with 25 ng/ml NGF alone. It is worth noting that the neurite outgrowth promoting and supporting effects of MC14 were also observed at relatively low concentrations of NGF. Significant neurite outgrowth enhancement was observed at concentrations of NGF as low as 1.3 ng/ml compared with the NGF-only control. Surprisingly, MC14 appeared to show neurotrophic effect in chronic treatment as 18% of neurite-bearing cells were recorded in the culture treated with MC14 alone. These results demonstrated that MC14 could significantly enhance the neurite outgrowth from PC12D cells and subsequently maintain and support the neurite outgrowth in long-term incubation period.

4-2-5. Mechanisms of action of MC14

The mechanisms of neurite outgrowth promoting activity of MC14 to PC12D cells were investigated in the intracellular signaling transduction level. Several representative intracellular signaling cascades were examined by the utilization of specific inhibitors.

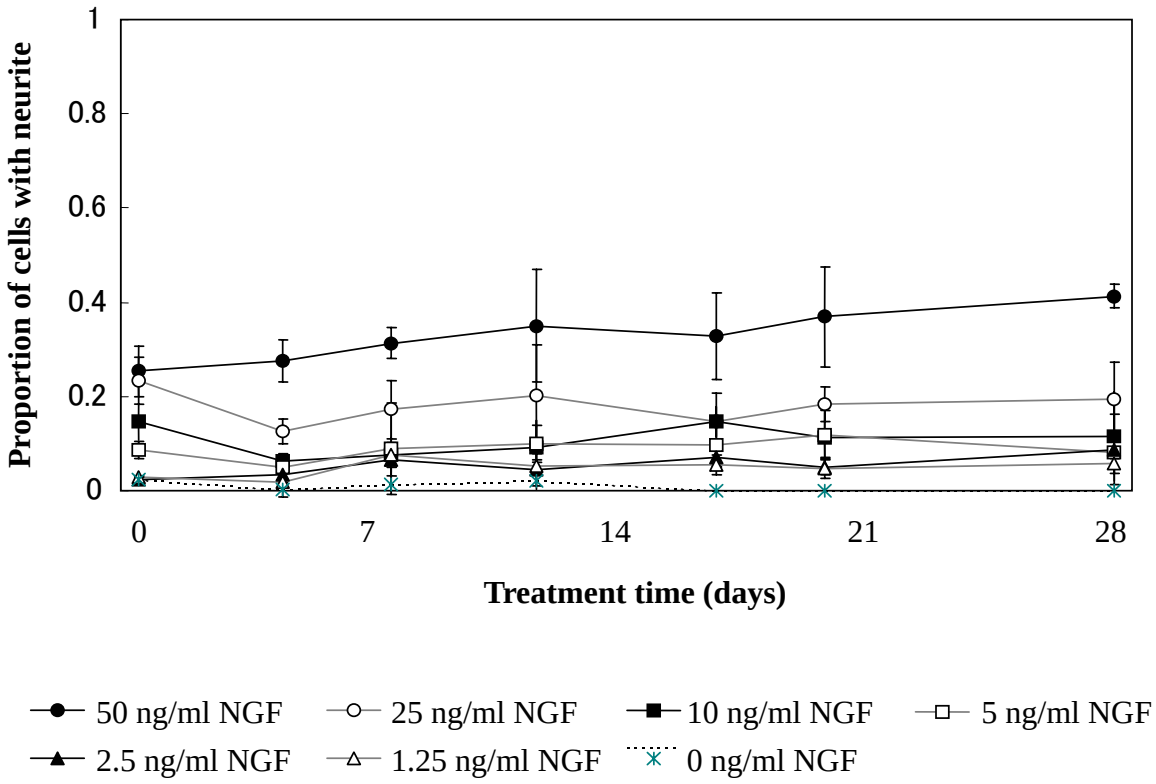


Fig. 4-6. Long-term effect of NGF on PC12D cells. Cells were treated with 0-50 ng/ml NGF for 28 days. The medium was renewed every 3 days to maintain the concentration of NGF during the incubation period. The medium was renewed every 3 days to maintain the concentration of MC14 and NGF during the experiment. Each datum point represents the mean ± SD (n=6).

(1) Effect of general protein kinases inhibitor on MC14-enhanced neurite outgrowth from PC12D cells

A general protein kinase inhibitor K252a was tested on its effect of NGF-induced and MC14-enhanced neurite outgrowth from PC12D cells. The neurite outgrowth inhibitory effect of K252a was examined at concentrations ranging from 1 to 64 nM on PC12D cells induced with 10 ng/ml and 50 ng/ml NGF. The lowest effective concentration of K252a was respectively 8 nM and 16 nM to significantly inhibit 50 ng/ml and 10 ng/ml NGF-induced neurite outgrowth from PC12D cells ($P < 0.05$, Student's *t*-test) (Fig. 4-7). Since K252a is a general protein kinase inhibitor, the effective inhibition to protein kinases is dependent on the concentration of K252a to the cell cultures. For example, it has been reported that 3 nM K252a could significantly inhibit the activity of protein kinase A receptor (TrkA) while PKA and PKC would be inhibited at concentrations of K252a higher than 25 nM. In order to specifically analyse the role of Trk receptor and also examine the involvement of protein kinases for the MC14-enhanced neurite outgrowth from PC12D cells, a wide concentration range of K252a was tested. As shown in Fig. 4-8, significant reduction of neurite outgrowth from PC12D cells was observed for the cultures pretreated with 8 nM K252a compared with the cultures treated with MC14 and NGF only (the inhibitor-untreated control). Approximately 40% of the number of neurite-bearing cells was reduced when the cells were pretreated with 16 nM K252a. Pretreatment of cells with 64 nM K252a completely blocked the combined effects of MC14 and NGF on PC12D cells as no neurite-bearing cells were observed under this concentration level of K252a.

(2) Effect of mitogen-activated protein kinase (MAPK) kinase inhibitor on

MC14-enhanced neurite outgrowth from PC12D cells

PD98059, a well-known specific MAPK kinase (or MEK) inhibitor, has been reported to inhibit the MAP kinases activity in PC12 cells. In order to determine the optimal effective concentration of PD98059 for inhibiting the NGF-induced neurite outgrowth from PC12D cells, cells were preincubated at concentrations of PD98059 ranging from 0 to 100 μM for 1h before MC14 and NGF were added. Neurite outgrowth of PC12D cells after pretreatment of cells with PD98059 in the presence of 10 ng/ml and 50 ng/ml NGF are shown in Fig. 4-9. Pretreatment of cells with PD98059 at 0.1-100 μM did not significantly inhibit the neurite outgrowth of cells induced by 10 ng/ml NGF. However, substantial inhibition of neurite outgrowth by PD98059 was observed for the cultures induced by 50 ng/ml NGF. Approximately 50% inhibition of neurite outgrowth was recorded by the pretreatment with PD98059 at 1-10 μM. Since these data were comparable to the effect of PD98059 on PC12 cells as reported elsewhere (Pang *et al*., 1995). Therefore, the inhibitory effects were determined at 3 μM or 10 μM PD98059.

Effect of PD98059 at 3 μM and 10 μM on MC14-enhanced neurite outgrowth from PC12D is shown in Figs. 4-10 and 4-11, respectively. Pretreatment of 3 μM PD98059 substantially blocked the neurite outgrowth enhanced by 1.5 μg/ml and 3 μg/ml MC14 (Fig. 4-10). At 10 μM PD98059, complete inhibition of the neurite outgrowth enhancement by 1.5 μg/ml MC14 was observed while 90% of neurite outgrowth induced by the combined effect of 3 μg/ml MC14 and 10 ng/ml NGF was blocked. Since no significant inhibition of neurite outgrowth induced by 10 ng/ml NGF could be observed within the test concentration of PD98059, the activation of MAP kinases may

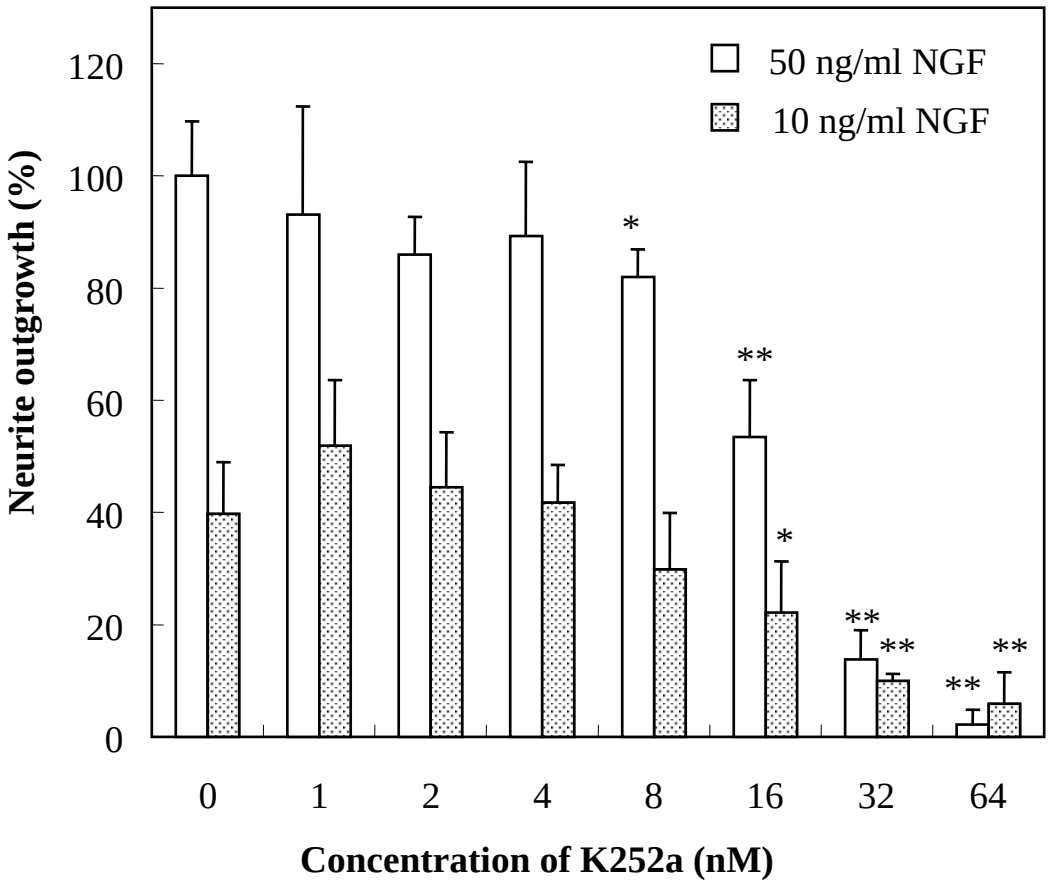


Fig. 4-7. The neurite outgrowth inhibitory effect of K252a on NGF-induced PC12D cells. Cells were pretreated with K252a for 1 h before the addition of 50 ng/ml or 10 ng/ml NGF and further incubated for 48 h. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the inhibitor-untreated control: ****P* < 0.05, *****P* **< 0.01 (Student's *t*-test).**

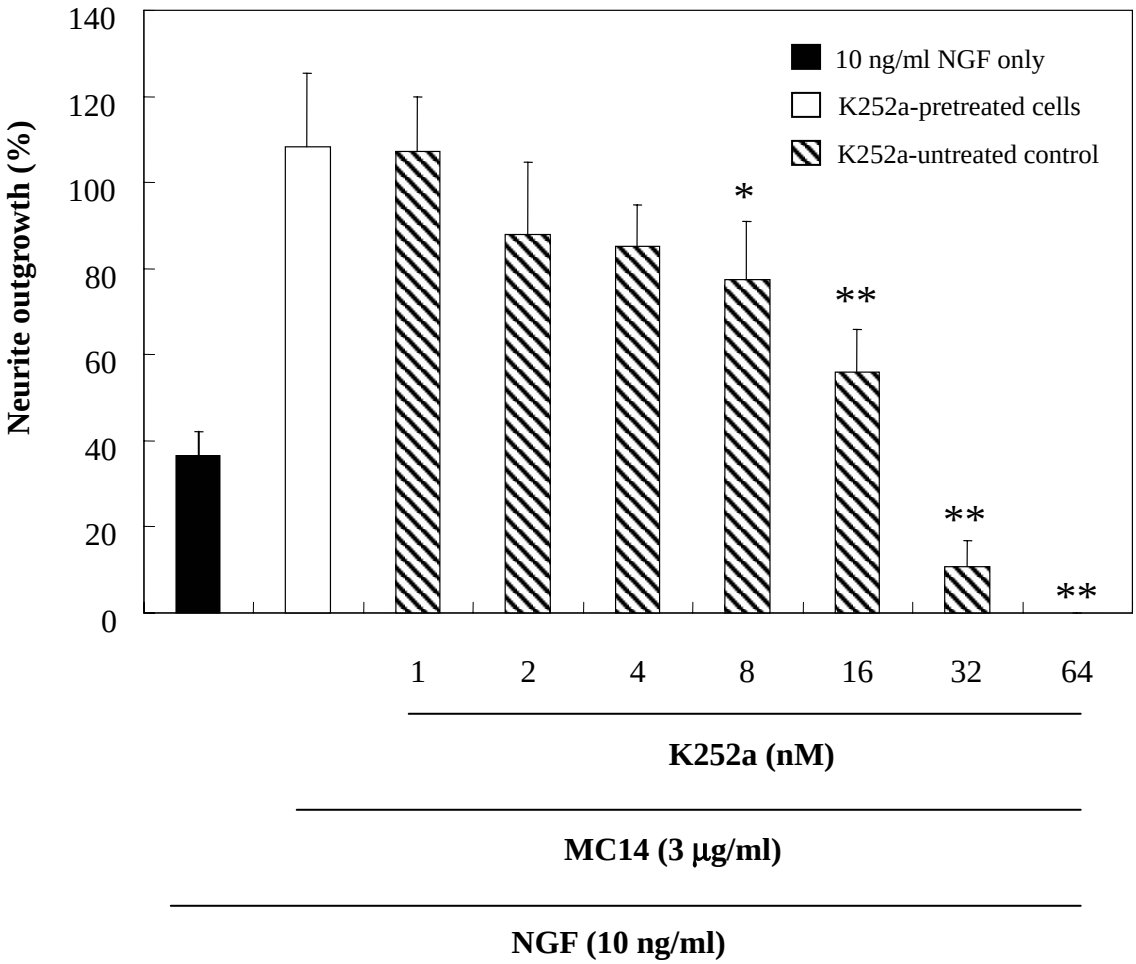


Fig. 4-8. Effect of K252a on neurite outgrowth promoting activity of MC14 on PC12D cells. Cells were pretreated with the indicated concentration of K252a for 1 hour before the addition of 10 ng/ml and 3 μg/ml MC14 and further incubated for 48 h. Closed bar represents the cells treated with 10 ng/ml NGF only. Open bar represents cells treated with MC14 and NGF only (inhibitor-untreated control). Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the inhibitor-untreated control: *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

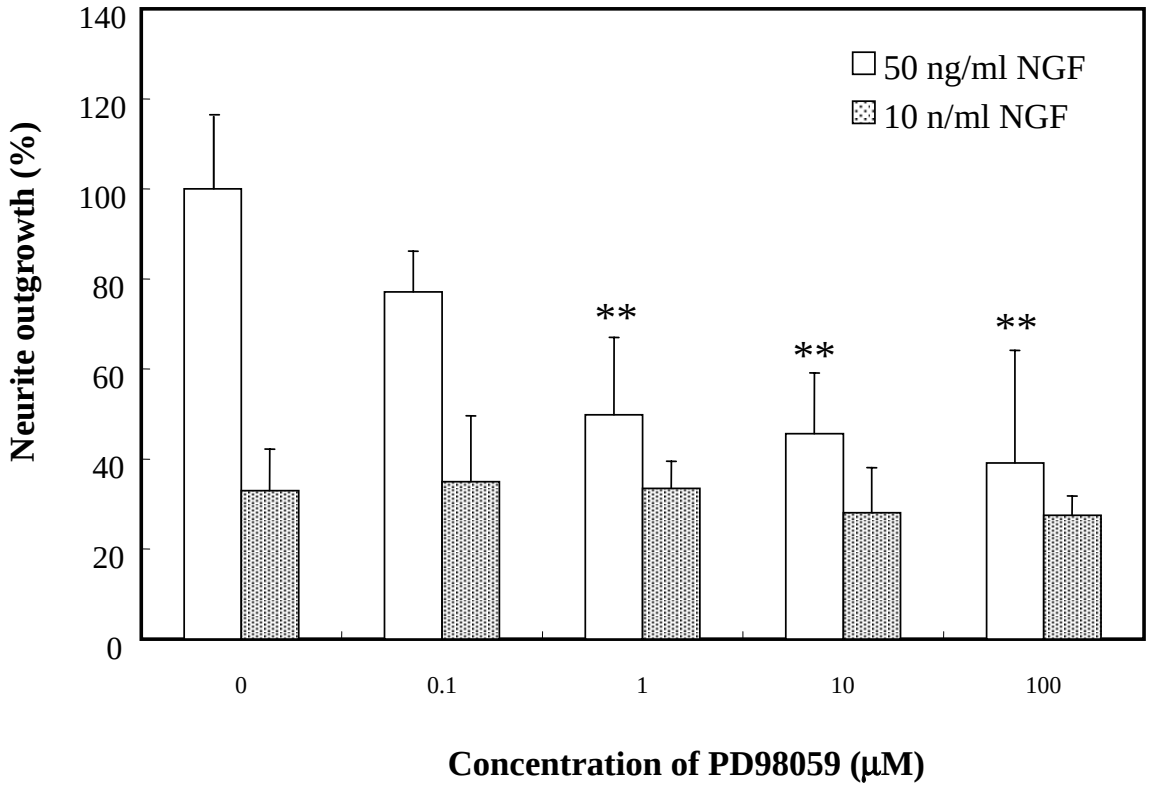


Fig. 4-9. The neurite outgrowth inhibitory effect of PD98059 on NGF-induced PC12D cells. Cells were pretreated with PD98059 for 1 h before the addition of 50 ng/ml or 10 ng/ml NGF and further incubated for 48 h. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the inhibitor-untreated control: *****P* **< 0.01 (Student's *t*-test).**

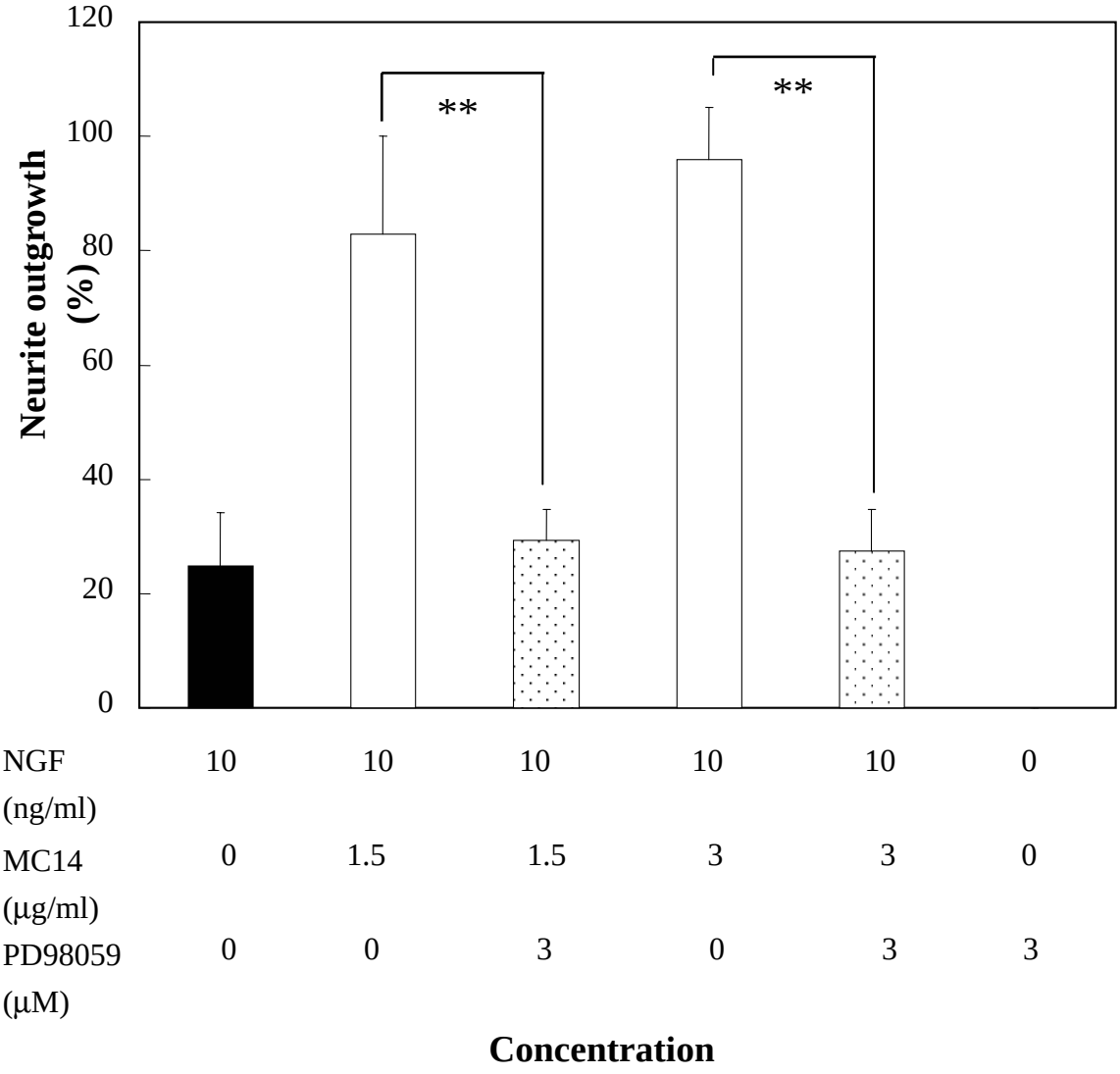
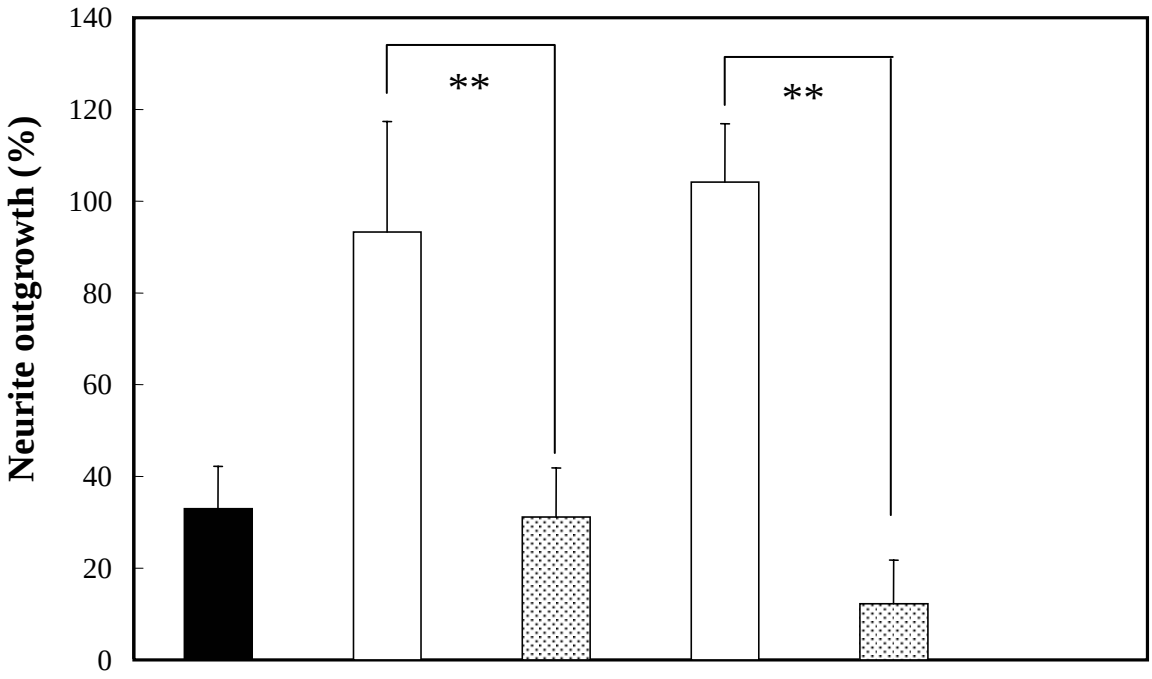


Fig. 4-10. Effect of PD98059 (3 μM) on neurite outgrowth promoting activity of MC14 on PC12D cells. Cells were pretreated with 3 μM PD98059 for 1 hour before the addition of 1.5 μg/ml or 3 μg/ml MC14, and 10 ng/ml NGF. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the inhibitor-untreated control: *****P* **< 0.01 (Student's *t*-test).**



NGF (ng/ml)	10	10	10	10	10	0
MC14 (μg/ml)	0	1.5	1.5	3	3	0
PD98059 (μM)	0	0	10	0	10	10

Concentration

Fig. 4-11. Effect of PD98059 (10 μM) on neurite outgrowth promoting activity of MC14 in PC12D cells. Cells were pretreated with 10 μM PD98059 for 1 h before the addition of 1.5 μg/ml or 3 μg/ml MC14, and 10 ng/ml NGF. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the inhibitor-untreated control: **$P < 0.01$ (Student's *t*-test).

not be necessary for the neurite outgrowth induced by low level of NGF (10 ng/ml). While substantial inhibition of neurite outgrowth from PC12D cells, which were treated with MC14 and 10 ng/ml NGF, by PD98059 suggested that the enhanced neurite outgrowth was due to the activation of MAP kinases-mediating signaling pathway, which appeared to be activated by MC14, rather than 10 ng/ml NGF.

(3) Effect of Protein Kinase A (PKA) inhibitor on MC14-enhanced neurite outgrowth from PC12D cells

Next, the effect of a cAMP-dependent PKA inhibitor was tested on MC14-enhanced neurite outgrowth from PC12D cells. As shown in Fig. 4-12, the pretreament with 4 nM PKA inhibitor significantly inhibited ($P < 0.05$, Student's *t*-test) the neurite outgrowth from PC12D cells induced by 50 ng/ml NGF while slight inhibition of the neurite outgrowth by 10 ng/ml NGF was observed at 100 nM of the PKA inhibitor. Therefore, 1, 10 and 100 nM of the PKA inhibitor were employed and their effects on the activity of MC14 were evaluated. Pretreatment of cells with 1 nM PKA inhibitor caused a 34% reduction of neurite outgrowth from PC12D cells compared with the inhibitor-untreated cultures (cells treated with 10 ng/ml NGF and 3 μg/ml MC14 only) (Fig. 4-13). The neurite enhancement by MC14 was completely blocked by 10 nM PKA inhibitor, suggesting that the MC14-enhanced neurite outgrowth from PC12D cells is likely mediated, at least partially, by the activation of PKA.

(4) Effect of Protein Kinase C (PKC) inhibitor on MC14-enhanced neurite outgrowth from PC12D cells

Pretreament of PC12D cells with the PKC inhibitor, chelerythrine chloride at

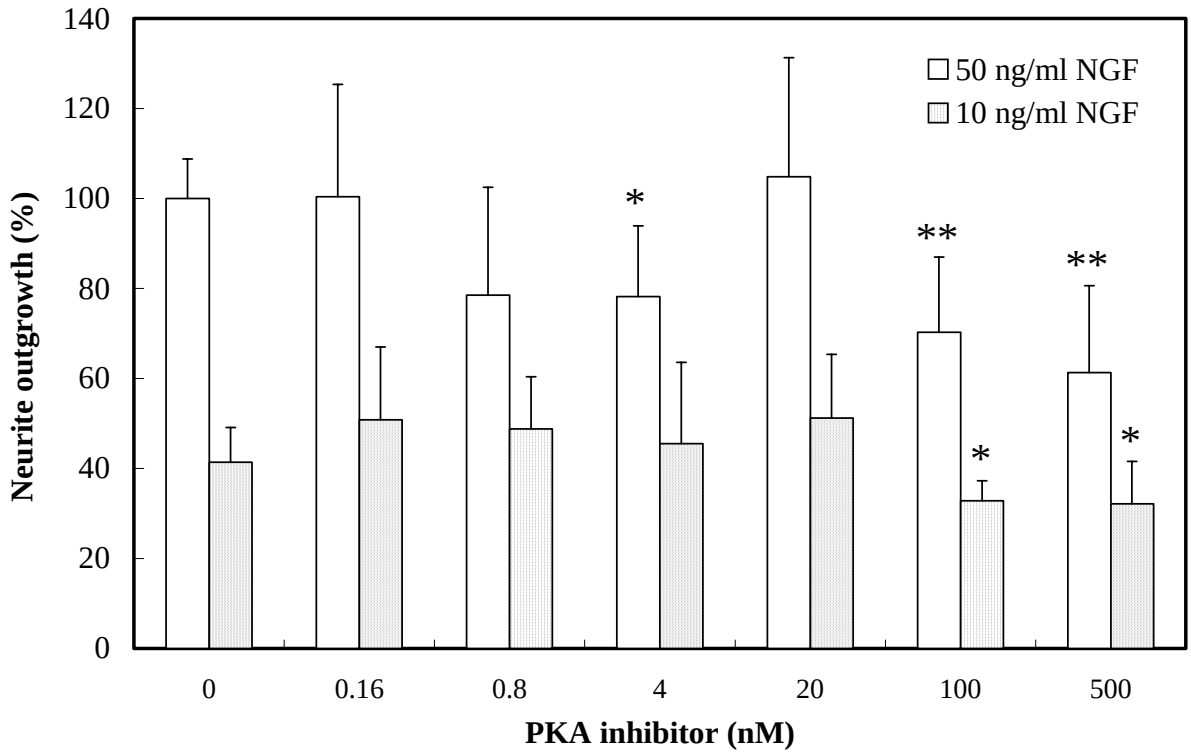


Fig. 4-12. The neurite outgrowth inhibitory effect of PKA inhibitor on NGF-induced PC12D cells. Cells were pretreated with PKA inhibitor for 1 h before the addition of 50 ng/ml or 10 ng/ml NGF and further incubated for 48 h. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the inhibitor-untreated control: *****P* **< 0.01 (Student's *t*-test).**

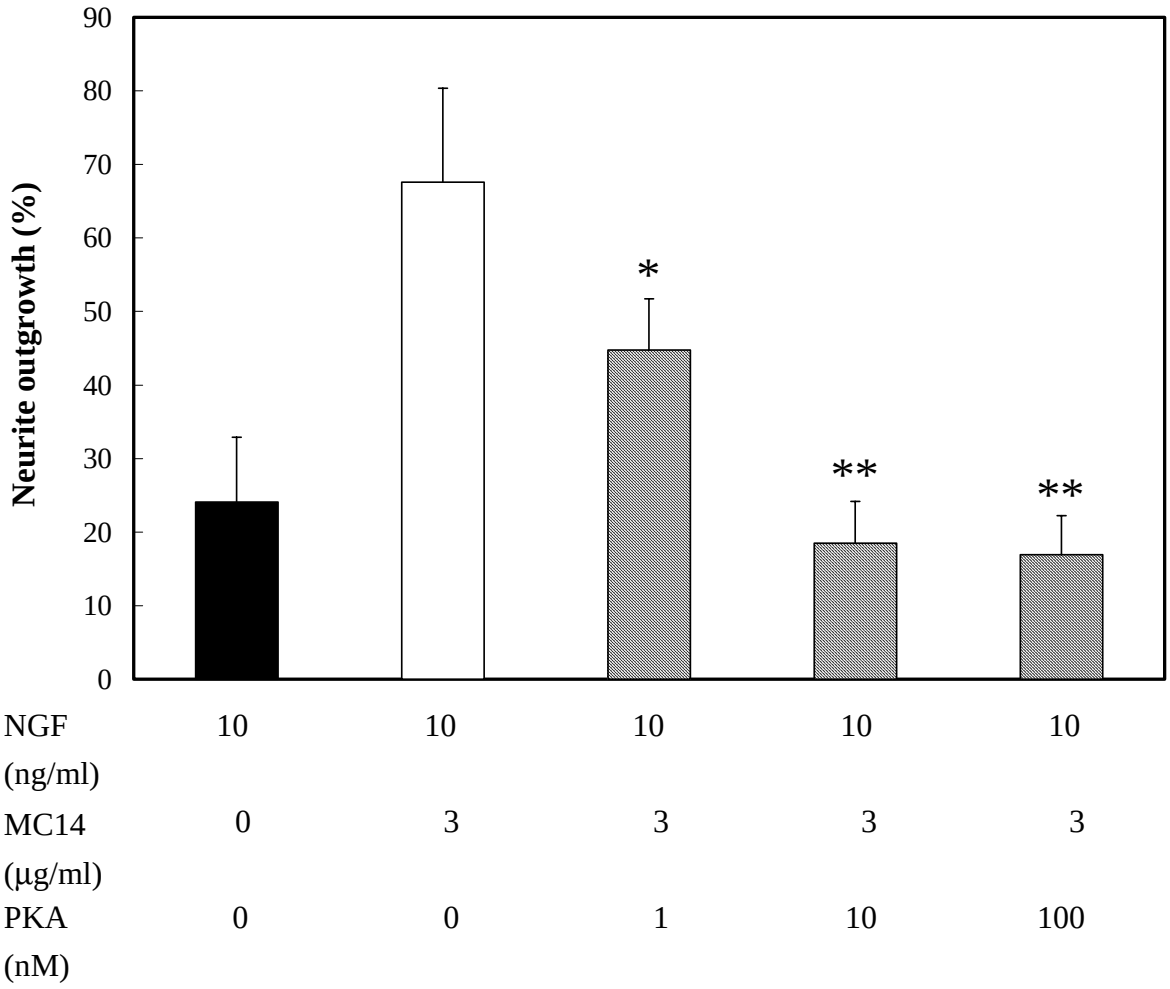


Fig. 4-13. Effect of PKA inhibitor on neurite outgrowth promoting activity of MC14 in PC12D cells. Cells were pretreated with PKA inhibitor for 1 h before the addition of 3 μg/ml MC14 and 10 ng/ml NGF. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments. Significant difference from the inhibitor-untreated control: *$P < 0.05$, **$P < 0.01$ (Student's *t*-test).

concentrations ranging from 0 to 9600 nM did not produce significant difference of the number of neurite-bearing cells from those of the inhibitor-untreated controls, both 10 ng/ml and 50 ng/ml NGF-induced cells (Fig. 4-14), indicating that the PKC inhibitor cannot effectively inhibit the neurite outgrowth induced by NGF on PC12D cells. To study the effect of this PKC inhibitor on MC14-enhanced neurite outgrowth, the reported effective concentration of chelerythrine chloride (IC_{50} = 660 nM) for inhibiting the PKC activity was taken as reference to determine its test concentration.

Compared with the neurite outgrowth of PC12D cells stimulated by MC14 and NGF, no significant neurite outgrowth inhibition was observed for the cultures pretreated with chelerythrine chloride at 600 nM (IC_{50} for PKC inhibition) or even 9600 nM (16-time of IC50) (Fig. 4-15). These data indicate that chelerythrine chloride did not inhibit the NGF-induced neurite outgrowth, as well as MC14-enhanced neurite outgrowth from PC12D cells.

4-3. Discussion

4-3-1. Neurite outgrowth promoting activity of MC14 on PC12D cells

PC12D cells have been considered as an ideal model system for studying the mechanism of NGF action as they response NGF by extending neurite and undergo neuronal differentiation (Katoh-Semba *et al*., 1987; Sano & Iwanaga, 1994). In addition, they can be used to evaluate the neurotrophic action of certain biologically active compounds, or the substances that could potentiate the effect of NGF, by determining their neurite outgrowth as a morphological parameter. The purified active substance (designated as MC14) isolated from the brown alga *S. macrocarpum*, significantly

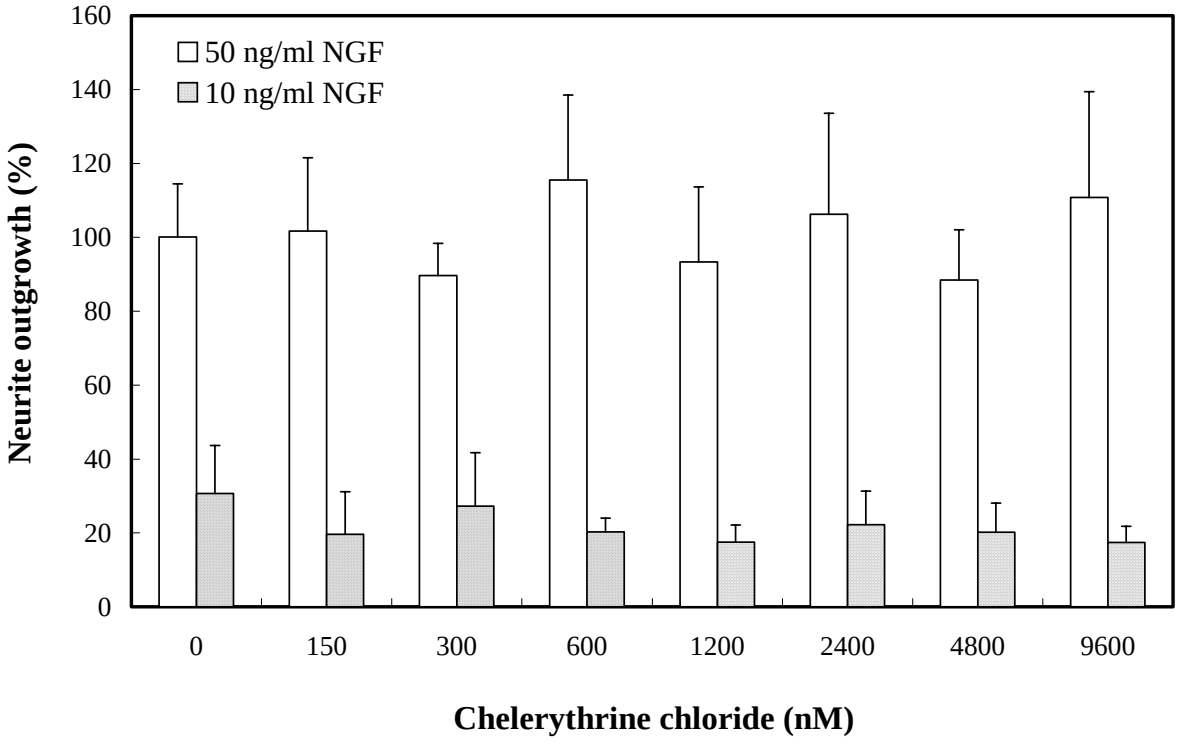


Fig. 4-14. The neurite outgrowth inhibitory effect of chelerythrine chloride on NGF-induced PC12D cells. Cells were pretreated with chelerythrine chloride for 1 h before the addition of 50 ng/ml or 10 ng/ml NGF and further incubated for 48 h. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments.

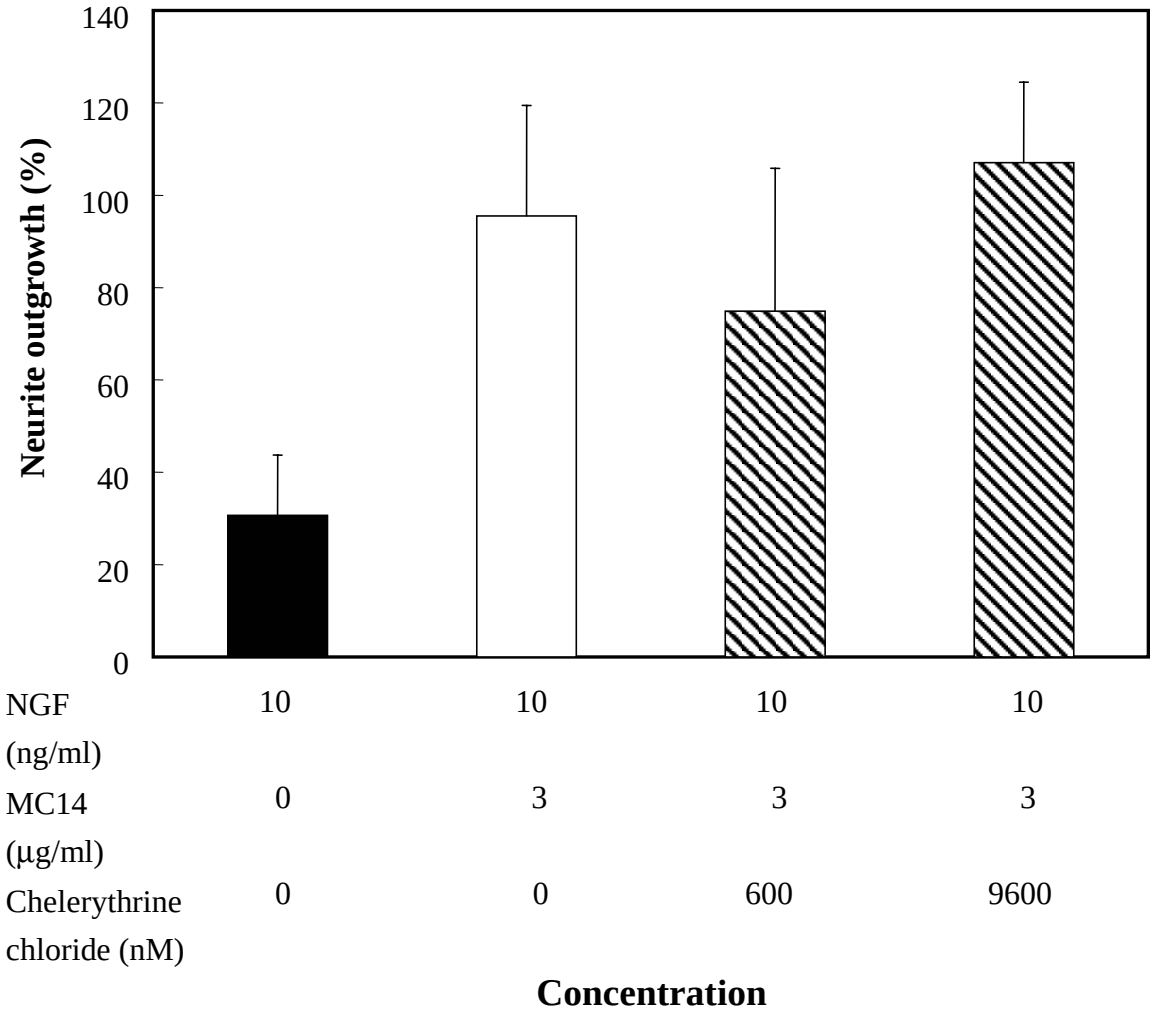


Fig. 4-15. Effect of chelerythrine chloride on neurite outgrowth promoting activity of MC14 in PC12D cells. Cells were pretreated with chelerythrine chloride for 1 h before the addition of 3 μg/ml MC14 and 10 ng/ml NGF. Neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD from two replicate experiments.

enhances neurite outgrowth from PC12D cells from a relative low NGF concentration to the saturated concentration of NGF, implying that it may be beneficial during the developmental stage of nervous system (Sagara *et al*., 1998). Moreover, as MC14 is a non-peptide molecule with a low molecular weight, it may readily access to the brain and modulate the action of NGF. Apart from neurogenesis, NGF is also essential to the development and maintenance of peripheral and central nervous systems. Numerous evidence from animal and clinical tests has already revealed that NGF possesses a potential for treating neurodegenerative diseases including Alzheimer's disease (Fischer *et al*., 1987; Ebendal *et al*., 1991; Olson, 1993; Seiger *et al*., 1993; Brinton & Yamazaki, 1998; Connor & Dragunow, 1998). However, the high molecular weight of NGF limits its therapeutic application because of its impermeability to the blood-brain barrier and susceptible to be metabolized by peptidase when administered peripherally (Olson, 1993). The present study provided the first line of evidence showing that the MC14 is a potent neurite outgrowth enhancer on PC12D cells, implying that it may also have the potential to enhance the action of NGF in terms of supporting the neuronal cell survival and rescuing the neuronal cell death caused by age-related diseases. The study of neuroprotective effect of MC14 will be discussed in detail in chapter V.

4-3-2. Chronic effect of MC14 on neurite outgrowth

The results of chronic effect of MC14 on PC12D cells clearly demonstrated that MC14 could significantly enhance the neurite outgrowth from PC12D cells at the early period of treatment and subsequently maintain and support the neurite outgrowth in long-term period of treatment. More importantly, MC14 promotes the construction of network-like neuritic connection between the neuronally differentiated PC12D cells,

suggesting that MC14 does not only promote the initial neurite extension from the cell body, but also facilitate the construction of neuritic network, implying that the pharmacological treatment of MC14 might promote NGF to form the functional synaptic connection among the neuronal cells during developmental stage, or functional synaptic reconstruction after axonal injury in the nervous system. Further study of the neurite regenerating effect of MC14 will be discussed in chapter VI.

4-3-3. Mechanisms of the neurite outgrowth promoting activity of MC14 on PC12D cells

Although the signaling cascades mediating the NGF-induced neurite outgrowth on the PC12 cells have been extensively studied, little is known about those in the sub-cell line of PC12 cells, PC12D cells. In this study, the signaling cascades activated by NGF on PC12D cells was characterized, and the mechanisms of action of MC14 was elucidated by analysing the signal transduction pathways involved in the neurite outgrowth promoting action of MC14 on PC12D cells.

As intracellular signaling cascade is initiated by the interaction of ligand-receptor binding, the involvement of the NGF receptor TrkA in MC14-stimulated cells was studies first. The result indicates that activation of TrkA receptor is required for the NGF-induced neurite outgrowth from PC12D cells as significant inhibition of neurite outgrowth was observed when TrkA receptors had been inactivated by 8 nM of K252a (an inhibitory concentration for the TrkA activity). Hence, neurite outgrowth of PC12D cells is initiated by NGF-TrkA interaction, like the case in the PC12 cells. In addition, the results demonstrate that intracellular protein kinases have an essential role in regulating the neurite outgrowth from PC12D cells as a complete inhibition of neurite

outgrowth was observed by treating the cells with K252a at 64 nM, which is the inhibitory concentration for protein kinases. Due to the observation that K252a at a TrkA-inhibiting concentration (8 nM) could also slightly but significantly inhibit the action of MC14, it may enhance the neurite outgrowth from PC12D cells, at least partially, by promoting the activation of a greater number of NGF receptors so that NGF-receptor mediated intracellular signaling pathway is amplified. This may be probably achieved by facilitating the binding of NGF to TrkA receptor. Besides, the action of MC14 is mediated by the intracellular protein kinases as no neurite-bearing cells could be found when total protein kinases were inactivated by 64 nM K252a.

In order to identify the downstream signaling molecules involved in the NGF-receptor-mediated signaling cascade, MAP kinase is studied as it regulates many important neurotrophic actions of NGF such as neuronal differentiation. Results of PD98059 inhibition tests revealed that MAP kinases-mediating signaling pathway can only be activated by high concentration of NGF. Besides, this signaling pathway can be activated by MC14 even in low concentration of NGF. Since the treatment of PD98059 at its MAPK effective inhibiting concentration completely blocks the MC14-enhanced neurite outgrowth, the MAP kinases-mediated signaling cascade should be the major pathway regulating the MC14 action on PC12D cells. Comparing to the result of Sano *et al* (1998) who showed that the activation of MAP kinases is not necessary for the neurite outgrowth from PC12D cells, our finding also demonstrated that PD98059 has no observable inhibitory effect on 10 ng/ml NGF-induced neurite outgrowth from PC12D cells. However, our data show that activation of MAP kinase is required for the morphological differentiation of PC12D cells induced by 50 ng/ml NGF. Furthermore, the NGF-induced neurite outgrowth in PC12D cells may involve more than one

pathway besides the NGF-TrkA-MAPK signaling cascade. One reason comes from the observation that neurite outgrowth can be induced by 10 ng/ml NGF under the inhibitory concentration of PD98059. Besides, inactivation of TrkA on MC14-stimulated PC12D cells only partially inhibited neurite outgrowth from PC12D cells. Therefore, MC14 may be able to separately activate MAPK kinase to complement the action of NGF. Nevertheless, it should be noted that MAP kinase is a key mediator to stimulate the MC14-enhanced neurite outgrowth from PC12D cells. Actually, numerous reports have demonstrated that MAP kinase-mediated signaling cascade is responsible for many important cellular processes induced by neurotrophic factors and other growth factors (Baeueries & Baltimore, 1988; Traverse *et al*., 1992; Pang *et al*., 1995; Dudley *et al*., 1995; Waters *et al*., 1995; Joneson *et al*., 1996; Hansen *et al*., 2000). This may be explained by the fact that MAP kinase is the major cytoplasmic protein kinase that can translocate into the nucleus, at which it activates various transcription factors for gene expressions, leading to differentiation, development or proliferation in different cell lines (Erikson, 1991). Based on these evidence, it is not surprising to find that the activation of MAP kinases is essential for the MC14-promoted neurtie outgrowth from PC12D cells. An interesting finding in this study is that PKA is also involved in the action of MC14 in PC12D cells, further support the diversified signaling cascades regulating the combined actions of MC14 and NGF in PC12D cells. PKA is generally known to be responsible for inhibiting apoptosis and its activation results in cell survival (Shirakawa & Mizel, 1989). The present finding implies that PKA may also be responsible for neurite outgrowth or differentiation in PC12D cells. In consistent with the finding by Katoh-Semba *et al* (1987), who reported that cAMP or cAMP-enhancing agents could induce the neurite outgrowth from PC12D cells, MC14

may amplify the production of cAMP by activating adenylate cyclase, or directly activate PKA, leading to the enhancement of neurite outgrowth from PC12D cells.

As PKC activation has been shown to enhance the neurite outgrowth via Ras-MAP kinase pathway after the stimulation of neural cell adhesion molecular or fibroblast growth factor (Hundle *et al.*, 1995; Huang *et al.*, 1995; Burry, 1998; Kolkova *et al.*, 2000). However, the present results indicate that PKC is neither involved in the NGF-induced nor MC14-enhanced neurite outgrowth from PC12D cells. This result implies that NGF cannot bind to the receptor of PKC-mediated signaling pathway, and MC14 is not capable of activating the PKC or its up-stream kinases.

Taken together, the striking finding in this study is that at least two independent signaling pathways are activated for the MC14-stimulated neurite outgrowth from PC12D cells, the TrkA-MAP kinase signaling cascade and the cAMP-dependent PKA signaling pathway. These two signaling pathways may be activated by MC14 individually to complement the action of NGF. Alternatively, MAP kinase is probably a downstream-effector of PKA-mediated signaling cascade because of the fact that MAP kinase is an important mediator protein kinase to integrate the complicated networking system and various extracellular stimuli in order to ‘fine- tune’ the network and, more importantly, to allow cross talk between the kinase pathways.

To summarize the results obtained from this part of study , the signaling pathways initiated by NGF in PC12D cells leading to neurite outgrowth is depicted in Fig. 4-16, and the proposed mechanisms of the action of MC14 in enhancing the neurite outgrowth from PC12D cells is shown in Fig. 4-17. The results of this study will provide an important insight with respect to the understanding of the signaling pathways involved in the neurite outgrowth of PC12D cells induced by NGF, and more importantly, for the

further use of PC12D cells as a model system to investigate the mechanisms of action of other NGF-promoting substances. In addition, the mechanism of action of MC14 to enhance the neurite outgrowth was suggested. These information would be equally important for the elucidation of biochemical change taken place in PC12D cells in response to NGF and MC14.

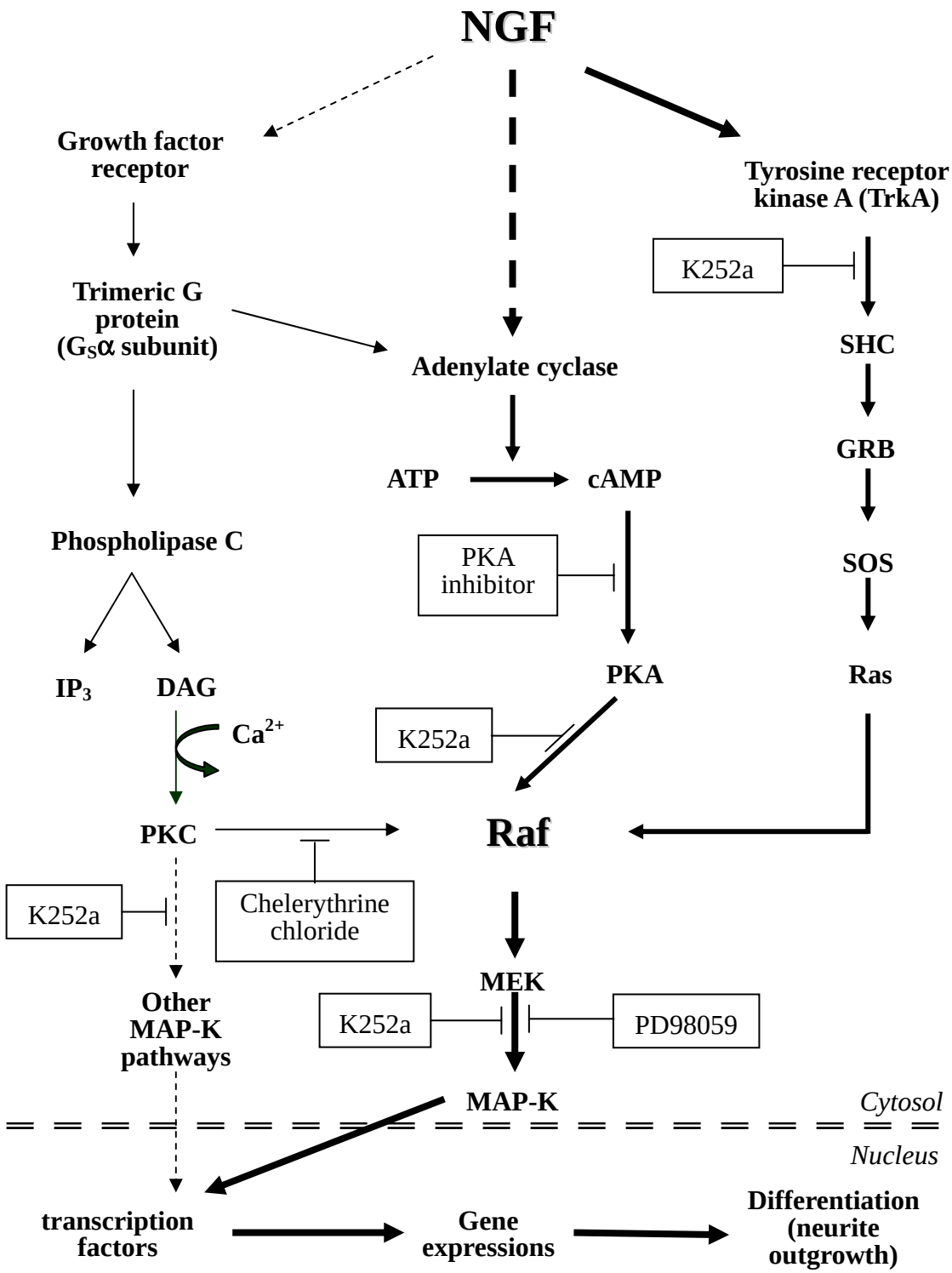


Fig. 4-16. Schematic representation of signaling pathways involved in NGF-induced neurite outgrowth from PC12D cells. See next page for caption.

Fig. 4-16 (cont'). Binding of NGF to TrkA receptor results in activation of a series of signaling proteins, including a group of GTP-binding proteins SHC, GRB, SOS and Ras. Subsequent, Ras protein phosphates Raf, resulting in enhanced mitogen-activated protein kinase kinase (MEK) phosphorylation and activation, which promotes dual threonine/tyrosine phosphorylation of mitogen-activated protein kinase (MAP-K), an event critical for stimulation of MAP-K activation. NGF may also activate adenylate cyclase by an unknown mechanism that, in turn, stimulate the conversion of ATP to cyclic 3', 5'-adenosine monophosphate (cAMP), which activates cAMP-dependent protein kinase A (PKA). It is expected that PKA can phosphorylate several different kinase including Raf, leading to the activation of MAP-K via MEK. MAP-K then translocates into nuclus and phosphorylate several transcription factors that elicit gene expression for neurite outgrowth and differentiation in PC12D cells. The proposed pathways elicited by NGF in PC12D cells leading to neurite outgrowth is indicated by the thick solid arrows. The unknown pathway is indicated by broken arrow. The point of action of inhibitors is indicated in T-line.

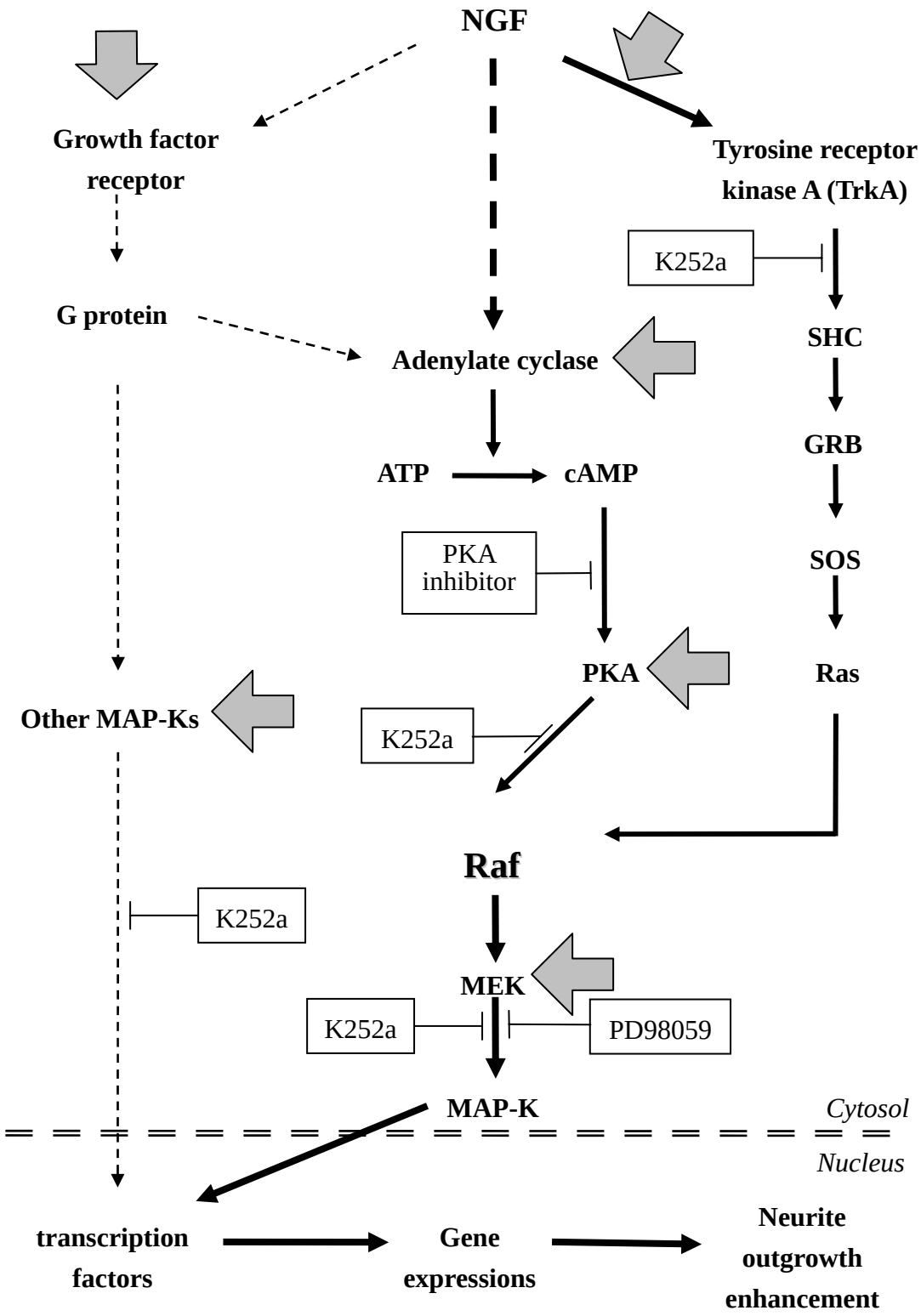


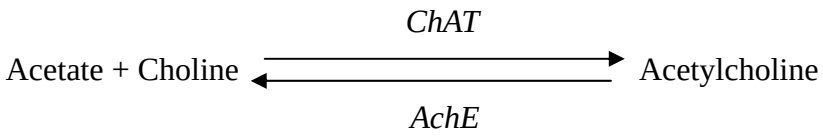
Fig. 4-17. Schematic representation of signaling pathways involved in MC14-enhanced neurite outgrowth from PC12D cells. See next page for caption.

Fig. 4-17 (cont'). Schematic representation of signal transduction pathways activated by MC14 in the presence of NGF in PC12D cells. The potential acting points of MC14 on the signaling pathways in PC12D cells induced by NGF-dependent or –independent intracellular signaling pathways are indicated by the gray think arrow. At receptor level, MC14 may facilitate the binding of NGF to TrkA receptor on the PC12D cells. Other surface receptor such as growth factor receptor or G-protein couple receptor may be directly activated by the interaction with MC14 resulting in the activation of certain MAP-K signaling cascade. In addition, MC14 activates PKA or its up-stream protein kinase such as adenylate cyclase. MEK-mediated signaling pathway is likely to be mainly amplified by MC14 leading to the enhancement of neurite outgrowth from PC12D cells. The unknown pathway is indicated by broken arrow. NGF-mediated signaling pathways are shown in solid arrow.

Chapter V

Acetylcholinesterase Promoting Activity of MC14

Acetylcholine (Ach) is a well-studied neurotransmitter in the nervous system that is released from the synaptic terminals of cholinergic neuron. Acetylcholine is synthesized by the enzyme choline acetyltransferase (ChAT) from the precursor choline and acetate. After finishing its job as a neurotransmitter, acetylcholine is inactivated by being split into choline and acetate by the enzyme acetylcholinesterase (AchE) as shown in the following equation (Matthews, 1998):



ChAT and AchE are the key enzymes for maintaining the normal function of acetylcholine as a neurotransmitter, and their expressions represent the functional differentiation of neurons (Das & Barone, 1999). One of the action of NGF during the early developmental stage is to induce the expression of these enzymes in cholinergic neurons (Barde, 1994).

On the other hand, it has been reported that NGF significantly increases the AchE

activity in PC12 cells ((Rieger *et al*., 1980; Greene & Rukenstein, 1981). Because this enzyme is present in PC12 cells grown without NGF and, unlike choline acetyltransferase, has a specific activity that does not vary as a function of cell density, the assessment of AchE in PC12 cells has been widely used as a biochemical marker to evaluate the NGF-induced functional differentiation of PC12 cells (Rieger *et al*., 1980; Katoh-Semba *et al*., 1987; Pradines *et al*., 1995). Greene & Rukenstein has reported that the effect of NGF on regulating AchE activity and neurite outgrowth in PC12 cells may be regulated via parallel or branching pathways (Greene & Rukenstein, 1981).

Accordingly, it is necessary to examine more closely whether MC14 can promote NGF-induced AchE activity in PC12D cells, in order to provide a biochemical evidence for MC14 to promote a functional differentiation of PC12D cells.

5-1. Materials and Methods

5-1-1. Materials

Sodium chloride solution (0.15 mol/l, supplemented with chloroform), *m*-nitrophenol, acetylcholine chloride and acetic acid were purchased from Sigma. Total protein content of each sample was determined by Micro BCA Protein Assay Reagent Kit from Pierce.

5-1-2. Cell culture

PC12D cells were seeded on 6-well tissue culture plate (2 X 10^6 cells per well) coated with collagen in complete medium for 24 h. For the time-course study of

NGF-induced AchE activity in PC12D cells, medium was then changed to fresh complete medium containing various concentrations of NGF, the cultures were incubated at 37°C for 12, 24, 48, 72 and 96 h. For the study of dose-response activity of MC14, medium was changed to fresh complete medium containing NGF with or without MC14, the cultures were incubated at 37°C for 72 h. Cells were harvested and washed with ice-cold PBS twice to remove the serum (which may contain AchE). Cell pellets were collected by centrifugation at 1000 rpm for the determination of AchE activity.

5-1-3. Preparation of cell lysate

Cells were resuspended in 200 μl ice-cold Tris-HCl (20 mM, pH 7.5) containing 0.5% Triton X-100, 10 mM $MgCl_2$ and 150 mM NaCl, and lysed by brief sonication on ice-bath. For protein concentration determination, 50 μl of the each cell lysate sample was reserved and stored in 4°C before protein assay. The rest of sample was assayed for AchE activity.

5-1-4. Assay of acetylcholinesterase activity

AchE activity in each lysate sample was determined according to the instruction manual of Sigma AchE assay kit with minor modification. Briefly, cell lysate (20 μl each) was transferred into two 1.5 ml plastic tubes labelled ‘test’ and ‘blank’. Twenty μl of NaCl solution (0.15 M) was added into each tube. The ‘blank’ was incubated in 60°C water bath for 10 min. Samples in both ‘test’ and ‘blank’ were added with 300 μl water, 200 μl *m*-nitrophenol solution (0.75 g/l in 0.02 M phosphate buffer, pH 7.8) and acetylcholine chloride solution (150 g/l) sequentially. After mixing, the solution was

incubated in 25°C water bath one by one with one min interval. After 30 min, the absorbance at 420 nm of the solution was measured by spectrophotometer (Jusco, V-550). The difference of absorbance (ΔAbs) from 'test' and blank' was calculated for each cell lysate sample as follow:

$$\Delta Abs = Abs_{(BLANK)} - Abs_{(TEST)}$$

5-1-5. Calibration

To the 300 μl pooled cell lysate, 300 μl NaCl solution (0.15 M) was added and incubated in 60°C water bath for 10 min. Standard of acetic acid solution were prepared by serial diution with the stock acetic acid solution (0.02 M) and the inactivated cell lysate as described in the instruction manual. The absorbance of each standard acetic acid solution was recorded at 420 nm. Calibration curve was plotted of the difference of absorbance between each standard acetic acid solution and water (blank), versus the corresponding AchE activity as stated in the instruction manual. The AchE activity of each lysate sample was determined from the calibration curve. The specific AchE activity was calculated based on the protein concentrations in the enzymatically assayed sample determined with the Micro BCA Protein Assay Reagent Kit (Pierce). One Rappaport unit was defined as the amount of cholinesterase which will liberate one micromole of acetic acid from acetylcholine in 30 min at 25°C at pH 7.8.

5-1-6. Statistical analysis

Each datum point is the mean ± SD (n=3). Significant difference from the control was determined by the Student's *t*-test. $P < 0.05$ was considered to be a significant

difference.

5-2. Results

5-2-1. Calibration curve

The AchE determination was based on the use of an indicator m-nitrophenol to measure the acetic acid produced by the enzymatic hydrolysis of acetylcholine (Rappaport et al., 1959). AchE catalysed the hydrolysis of cholinesterase of various short chain organic acids, including acetylcholine, which is that substrate used in the assay.

$$\text{Acetylcholine} + H_2O \xrightarrow{\textit{AchE, pH 7.8}} \text{Acetic acid} + \text{Choline}$$

By conducting the reaction in the presence of the acid-base indicator (*m*-nitrophenol), the acetic acid produced lowers the pH, causing a loss of color. This color change is proportional to the AchE activity presented in the cell lysate sample. The blank is prepared by inactivating at 60°C for 10 min to compensate for background absorbance contributed by the sample. Absorbances of both ‘blank’ and ‘test’ were read at 420 nm and the absorbance difference was used to estimate the acetylcholinesterase level. The calibration curve used in the present experiment is shown in Fig 5-1. The pattern and scale of the calibration curve was very close to the reference proved by the instruction manual. Hence, it was used for determining the AchE activity of the cell lysate.

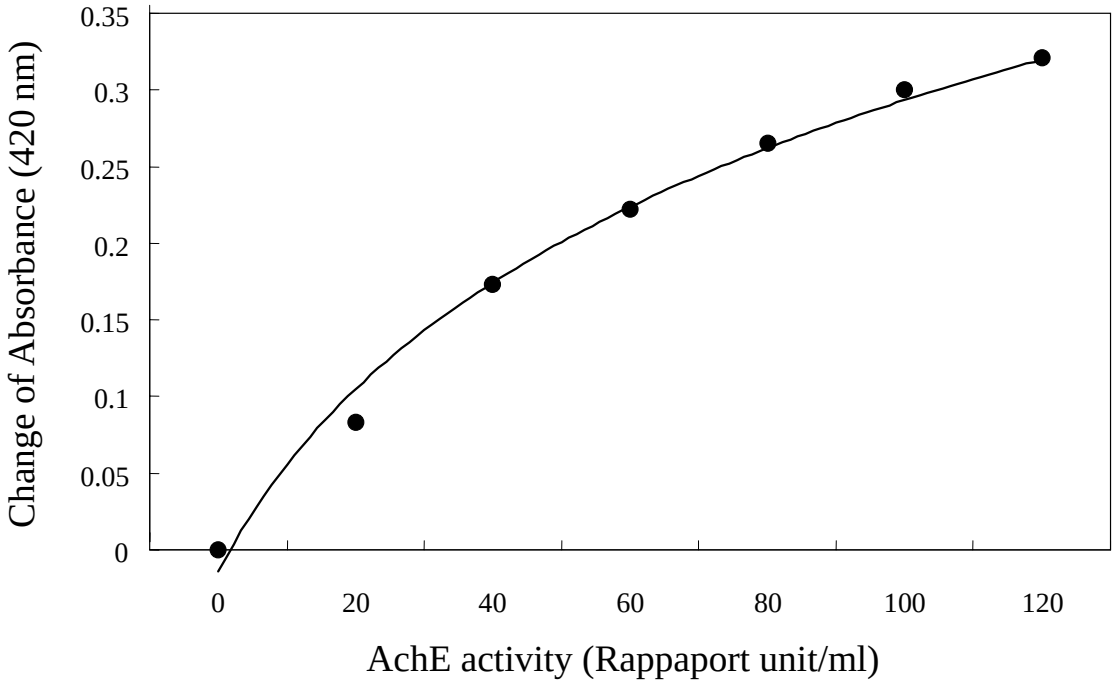


Fig. 5-1. Calibration curve for the determination of AchE activity in the lysate sample. Change of absorbance represents the difference of absorbance (420 nm) between sample and water (blank). The corresponding AchE activity is pre-determined by the AchE assay kit. One Rappaport unit is the amount of cholinesterase which will liberate 1 μmole of acetic acid from acetylcholine in 30 min at 25°C at pH 7.8 under conditions stated in the Methods and Materials.

5-2-2. Time profile of NGF-induced AchE activity in PC12D cells

The time profiles of the AchE activity in PC12D cells induced by 0, 10 and 50 ng/ml NGF are shown in Fig. 5-2. When the cells were treated in complete medium without NGF treatment, the cellular specific AchE activity remained at 0.2-0.28 rappaport unit/μg protein, indicating that the specific AchE activity in PC12D cells did not change with time throughout the experimental period. The specific AchE activity in PC12D cells increased steadily from the initial 0.28 rappaport unit/μg protein at 12 h to 0.44 rappaport unit/μg protein at 96 h, suggesting that AchE activity was slightly induced by the treatment of cells with 10 ng/ml NGF. However, the specific AchE activity in PC12D cells was substantially elevated after treating the cells in 50 ng/ml NGF for 72 h, at which a 2.7-fold enhancement was detected. At 96 h, the specific AchE activity dropped to 0.6 rappaport unit/μg protein. This may be explained by the reduction of cells' health condition due to the deficiency of nutrient and the accumulation of metabolic wastes in the medium. For further experiments, an optimal incubation time of 72 h was employed for determining the AchE promoting effect of MC14.

5-2-3. AchE promoting activity of MC14 on PC12D cells

To assess the promoting effect of MC14 on the NGF-induced specific AchE activity, PC12D cells were treated with NGF in the presence or absence of MC14 for 72 h. Table 5-1 shows that the significant enhancements of the specific AchE activity in PC12D cells were detected after the cells were treated with 3 μg/ml MC14 in 2 ng/ml NGF ($P < 0.05$, Student's *t*-test), 10 ng/ml NGF ($P < 0.01$, Student's *t*-test) and 50 ng/ml NGF ($P < 0.01$, Student's *t*-test). Treatment of cells with 3 μg/ml MC14 together with 2, 10 and 50 ng/ml NGF resulted in an 1.3-fold, 1.2-fold and 1.5-fold increase in the specific

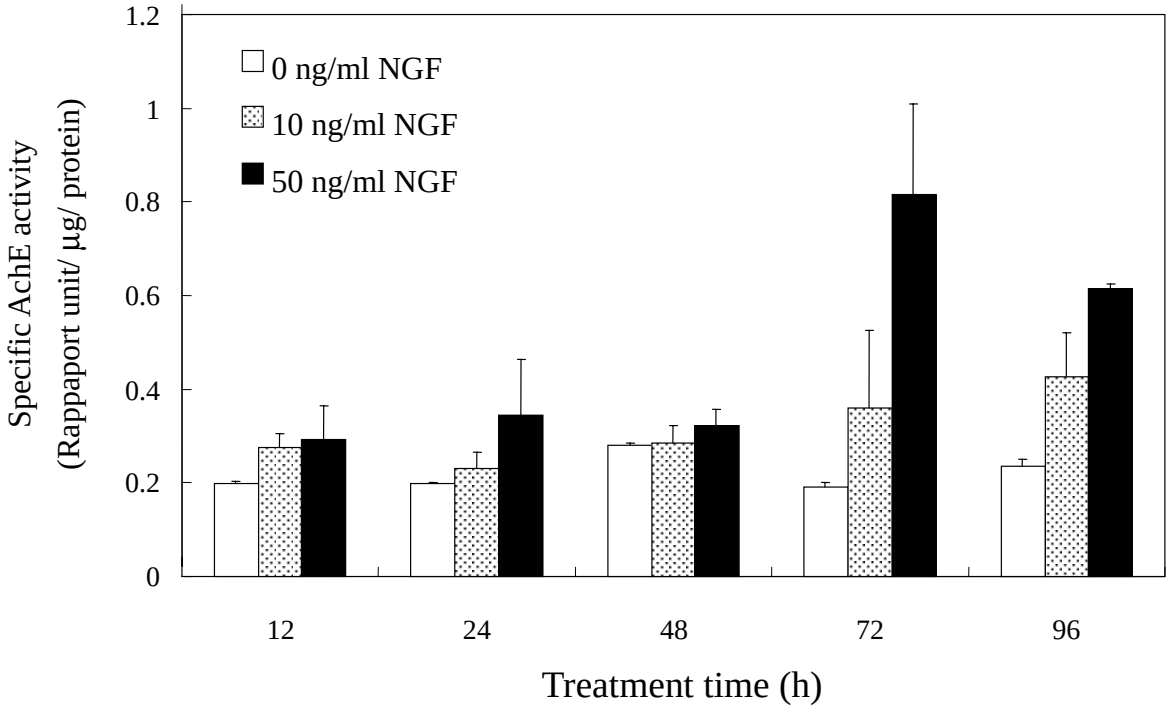


Fig. 5-2. Time-course AchE activity in PC12D cells induced by NGF. PC12D cells were treated with the indicated concentration of NGF for the indicated period. Specific AchE activity of the cell lysates was determined based on the protein concentration in the enzymatically assayed sample. Each datum point represents the mean ± SD (n=3).

Table 5-1. Acetylcholinesterase promoting activity of MC14

NGF *(ng/ml)*	**AchE** *(Rappaport unit/μg protein)*		***P*-value** *(Student's* **t-***test)*	
	NGF only	**MC14** *(3 μg/ml)*		
0	**0.692 ± 0.049**	**0.753 ± 0.110**	**0.428**	**(*P* > 0.05)**
2	**0.769 ± 0.060**	**0.994 ± 0.121***	**0.043**	**(*P* < 0.05)**
10	**0.871 ± 0.065**	**1.077 ± 0.061****	**0.007**	**(*P* < 0.01)**
50	**0.910 ± 0.100**	**1.322 ± 0.099****	**0.007**	**(*P* < 0.01)**

PC12D cells were incubated for 72 h in complete medium with the indicated concentrations of NGF in the presence or absence of MC14 (3 μg/ml). Each datum point represents mean ± SD (n=3). Significant difference from NGF-only control: ****P* < 0.05, *****P* **< 0.01 (Student's *t*-test) where *P* < 0.05 was considered to be a significant difference.**

AchE activity in PC12D cells, respectively. However, no significant change of the specific AchE activity in PC12D cells was observed when the cells were treated with 3 μg/ml MC14 only compared with the untreated control, suggesting the MC14 did not induce AchE activity in PC12D cells.

5-3. Discussion

Acetylcholine acts as an excitatory neurotransmitter for the cholinergic neurons in the central nervous system (CNS). Nuclei containing the cell bodies of cholinergic neurons are scattered throughout the brain, and cholinergic axons innervate most region of the CNS. Prominent sources of cholinergic inputs to the cortex and the hippocampus are nuclei in the basal forebrain, especially the septal nuclei and the nucleus basealis of meynert. Cholinergic neurons in these nuclei have widespread and diffuse projections, innervating the cortex, hippocampus and brain stem (Nicholls *et al*.,1992). Considerable evidence from both animal and human studies suggests that cholinergic systems are important for learning, memory, and cognition (Fibiger, 1991). Therefore, acetylcholine has an important role for these neuronal functions. On the other hand, for a functional synaptic transmission of nerve impulse, acetylcholine has to be degraded into choline and acetate by AchE, and about half of the choline molecule degraded from acetylcholine will be transported back to the cholinergic axon terminals for maintaining sufficient substrate for acetylcholine synthesis. Drugs that interfere with transmitter degradation or uptake can have profound effects on signaling, indicating that the rapid termination of transmitter action by AchE is crucial to synaptic function (Nicholls *et al*., 1992).

Present result indicates that MC14 can significantly promote the NGF-induced AchE activity in PC12D cells, demonstrating that MC14 can enhance the functional differentiation of PC12D cells. Besides, it provides additional biochemical evidence for the activity of MC14 to promote functional neurodifferentiation. Interestingly, several agents, namely EGF, dexamethasone and Bt_2cAMP, have been reported to modulate the phenotypic properties (e.g. neurite outgrowth) of PC12 cells neither affected their specific AchE activity nor significantly altered the response of AchE to NGF treatment (Edgar & Thoenen, 1978). The present finding clearly indicates that MC14 can promote both the NGF-induced morphological and enzymatical differentiation. However, MC14 does not mimic these actions of NGF.

The present result implies that MC14 may be beneficial to the normal development of the cholinergic nervous system associated with learning, memory and recognition.

Chapter VI

Neuronal Survival Supporting Activity of MC14

In chapters IV and V, the actions of MC14 on promoting neurite outgrowth and acetylcholinesterase activity in PC12D cells have been demonstrated. Both of these morphological and biochemical assessments support the MC14's capability of promoting differentiation on PC12D cells. These findings also provided evidence for the NGF-potentiating activity of MC14. Since NGF can induce diverse cellular effects, which are regulated through separate and distinct intracellular signaling pathways (Kimura *et al*, 1994; Yao & Cooper, 1995; Belliveau *et al*., 1997), it is necessary to further assess whether MC14 also promote NGF-induced survival support for exploring the potential of MC14 to treat neurodegenerative diseases such as Alzheimer's disease, which causes progressive neuronal cell death and loss of neurons (Whitehouse *et al*., 1982). Moreover, a number of reports have shown that some neuroactive agents such as forskolin and cAMP analogs do not exhibit both neuronal differentiating and neuroprotecive activities (Rukenstein *et al*., 1991; Ashcroft *et al*., 1999). These findings suggests that cellular responses such as neurite outgrowth and survival can be differentially regulated by different agents, making it necessary to further investigate whether MC14 also promotes survival supporting activity.

PC12 cell line has been extensively used as a modeling system to study the mechanisms of neuronal cell death because the neuronally differentiated PC12 cells depend NGF for survival in serum-free medium. Deprivation of NGF will cause the

neuronal PC12 cell death via apoptosis, an intracellular controlled process to cause cell death (Batistatou *et al*., 1991; Mesner *et al*., 1995). This characteristic has made PC12 cell line a very useful model system for the study of mechanisms of apoptosis as well as the neuroprotective effect of NGF on neuronal cells.

In this study, the survival supporting action of NGF on neuronal PC12D cells in serum-free medium was confirmed and the activity of MC14 to promote neuroprotection in the presence and absence of NGF were investigated. Cells viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which has been widely used as a sensitive and accurate method to measure cell viability (Denizot & Lang, 1986; Gerlier & Thomassel, 1986; Hansen *et al*., 1989; Vistica *et al*., 1991). The assay is designed for the spectrophotometric quantification of cell viability based on cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. This cellular reduction involves the pyridine nucleotide cofactors NADH and NADPH (Berridge & Tan, 1993). After the formazan salt crystals solubilized in the solubilizing solution and incubating overnight in humidified atmosphere, the resulting colored solution is quantified using a scanning multiwell spectrophotometric reader. The absorbance is proportional to the number of viable cells.

In addition to evaluate the survival supporting activity of NGF and promoting effect of MC14, their mechanisms of actions were investigated in order to get a thorough understanding of the signal transduction pathways regulating their actions. The second part of this chapter will focus on an in vitro study of neuroprotective effect of MC14 on PC12D cells against oxidative stress induced by hydrogen peroxide. This result would provide strong supportive pharmacological evidence for the potential application of

MC14 as a therapeutic agent to treat neurodegenerative diseases.

6-1. Survival supporting effect of MC14 on serum free-induced neuronal PC12D cell death

PC12 cell line has been extensively used as a modeling system to study the mechanisms of neuronal cells death because the neuronally differentiated PC12 cells depend NGF for survival in serum-free medium (Mesner *et al*., 1992). In order words, the deprivation of NGF will cause an intracellular controlled process for cell death. This characteristic has made PC12 cells a very useful cellular system for the study of the mechanisms of apoptosis (programmed cell death) as well as the neuroprotective action of NGF-promoting neuroactive substances. In this study, the survival supporting action of NGF on neuronal PC12D cells under serum-free condition for evaluating their potential application as a modeling system to study NGF-induced responses. Furthermore, the activity of MC14 to promote neuroprotection in the presence and absence of NGF were investigated. Finally, the mechanisms of action of MC14 was described to get a better understanding of its effect.

6-1-1. Methods and Materials

(1) Cell culture and preparation of neuronal PC12D cells

PC12D cells were maintained in complete medium as described in chapter 4. Neuronal PC12D cells were prepared as follow: PC12D cells ($5x10^6$ cells) were seeded on 10-cm tissue culture dish in complete medium for 24 h. For the ease of cell

detachment, the tissue culture dish was not coated with poly-L lysine. After 24 h incubation, medium was changed to a fresh medium containing DMEM supplemented with 5% FBS and 50 ng/ml NGF to induce differentiation. After 48 h, neuronal PC12D cells were harvested by trituration and washed in serum-free DMEM by three cycles of centrifugation-resuspension (Batitatou & Greene, 1991).

(2) Time-course study of serum-free induced neuronal PC12D cell death

The neuronal PC12D cells were plated on 96-well plate coated with poly-L-lysine at $5x10^3$ cells per well in serum-free DMEM. Cell viability was determined by MTT assay after an incubation period of 2, 4, 12, 24, 48 and 60 h.

(3) Study of NGF-induced survival supporting effect on neuronal PC12D cells

Neuronal PC12D cells were plated on poly-L-lysine coated culture plate as described above. For studying the dose-dependent survival supporting effect of MC14 on the neuronal PC12D cells under serum-free condition, NGF at a concentration range of 0-50 ng/ml was added to the cultures and incubated for 24 h. The absorbance value of cultures treated with 100 ng/ml NFG was assigned as positive control (optimal concentration for supporting cell survival in serum-free medium). The viability of other cell cultures was expressed as a percentage of positive control (100 ng/ml NGF, 100%) from the same experiment.

(4) Study of survival promoting and supporting activities of MC14 in the presence or absence of NGF

After neuronal PC12D cells were plated on 96-well plate as described above, NGF

and MC14 were added to the cell cultures. Concurrently, corresponding cell cultures were treated with NGF only for comparing the promoting effect of MC14. To assess the survival supporting effect of MC14, neuronal PC12D cells were treated with or without MC14. Cell viability was determined by MTT assay after incubated in serum free medium for 24 h.

(5) Effect of various inhibitors on MC14-stimulated neuroprotection on neuronal PC12D cells

After the neuronal PC12D cells were plated on 96-well plate as described above, aliquot of each inhibitor was added to cell cultures and incubated for 1 h before the additions of MC14. After a further 24 h incubation, the cell viability was determined by MTT assay. The following stock concentrations of inhibitors dissolved in the indicated solvent were used in the experiment: PD98059 (20 mM, DMSO); K252a (200 μM, MeOH); PKA inhibitor (5 mg/ml, DMEM) and chelerythrine chloride (0.5 mg/ml, DMEM). Each inhibitor was further diluted with serum-free DMEM to a suitable working concentration and aliquot of each inhibitor was added to the test well in less than 1% v/v of the medium, which has no effect to the cells. The inhibitors also have no effect on the cells incubated in serum free medium.

(6) MTT assay

Cell survival was evaluated by the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) (Hansen *et al*., 1989). MTT was added to the cultures at a final concentration of 0.5 mg/ml to each well. The plate was incubated for 4 h in a water-saturated atmosphere of 5% CO_2 at 37°C

before the addition of 100 μl solubilization solution (10% SDS in 0.01 M HCl). The plate was allowed to stand overnight in a water-saturated atmosphere at 37°C. After conforming the complete solubilization of the purple formazen crystals by microscopic observation, the spectrophotometrical absorbance was measured by a microtiter plate reader (BIO-RAD, Model 450). The absorbance at 570 nm and 655 nm were used as the main and reference wavelengths, respectively.

(7) Phase-contrast microscopy

The morphology of cells under various treatments in serum-free medium was recorded using a phase-contrast microscope with 200-400X magnification. Photo-slides were taken using a compatible camera (Oympus SC-35). Image was scanned from the slide and transformed into computer file using Polaroid Polarscan 25/LE system.

(8) Statistical analysis

Each datum point represents the mean ± S.D. (n=4-6). Significant difference from the control was determined by the Student's *t*-test. $P < 0.05$ was considered to be a significant difference.

6-1-2. Results

(1) Neuronal PC12D cell death induced in serum-free medium

As determined by MTT assay, cell viability significantly decreased after the neuronal PC12D cells were incubated in NGF-deprived serum-free medium for 6 h and more than 50% reduction was detected after 24 h (Fig. 6-1). At the end of the

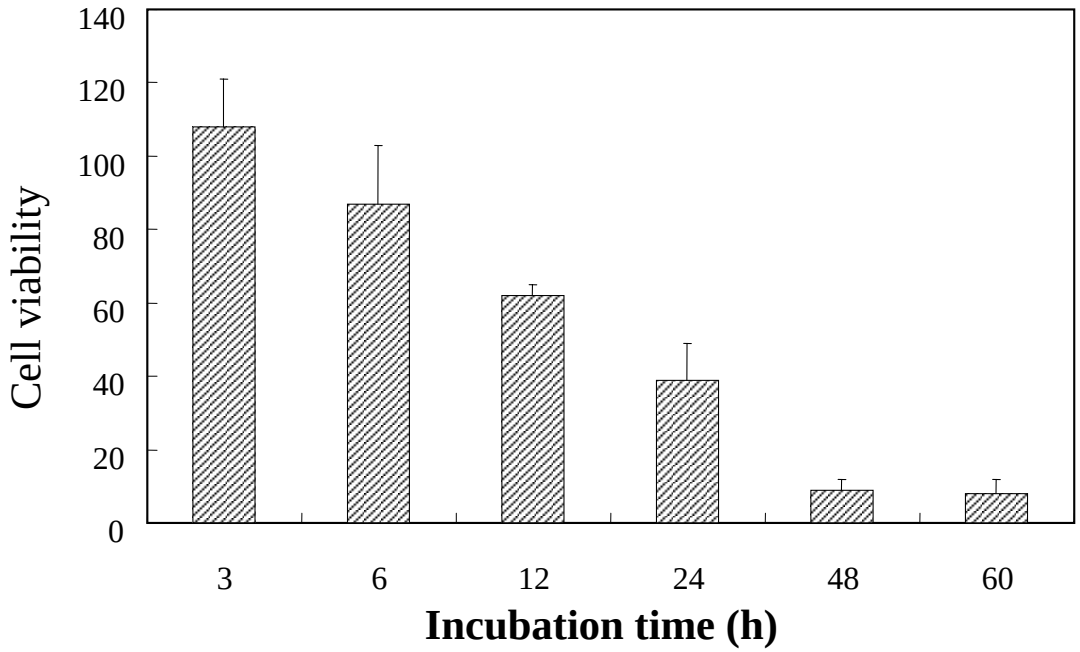


Fig. 6-1. Time-course neuronal PC12D cell death in serum-free medium. Neuronal PC12D cells were incubated in NGF-deprivated serum-free medium at the indicated time. Cell survival was determined by MTT assay, and expressed as a percentage relative to positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4) from two replicated experiments.

experimental period (60 h), cell viability was lower than 10% of positive control. Hence, an incubation time of 24 h was used as to examine the survival supporting activity of NGF and survival promoting effect of MC14 in the following experiments. With respect to the morphological change, increasing number of shrunk cells were observed after 6 h and almost all cell shrunk after 60 h of incubation in serum free medium (Fig. 6-2B).

(2) Survival supporting effect of NGF

NGF supported the survival of neuronal PC12D cells in serum-free medium in a concentration dependent manner (Fig. 6-3). At 100 ng/ml NGF, almost no shrunk cell was observed and some of the cells even extended long neurite (Fig. 6-2C), suggesting that NGF could effectively protect neuronal PC12D cells from serum-free-induced degeneration. Hence, the culture treated with 100 ng/ml NGF was assigned to be the positive control for the determination of cell viability.

(3) Survival promoting effect of MC14

The survival promoting effect of MC14 was first examined at 0.75 μg/ml MC14 in the presence of 0-100 ng/ml NGF. As shown in Fig. 6-4, slight but significant survival promoting effect of 0.75 μg/ml MC14 was detected in the presence of 0.02-50 ng/ml NGF. In an attempt to evaluate the optimal promoting effect, a high dose of MC14 together with NGF were added to the cultures. Treatment with 1.5 μg/ml MC14 further enhanced the percentage of viable cells by 25-40% over those treated with NGF only (Fig. 6-5). Both MC14 treatments exhibited NGF concentration-dependent survival promoting effect. In addition, the combined effect of MC14 and 50 ng/ml or 100 ng/ml NGF increased the cell viability to an extend higher than that of positive control, while

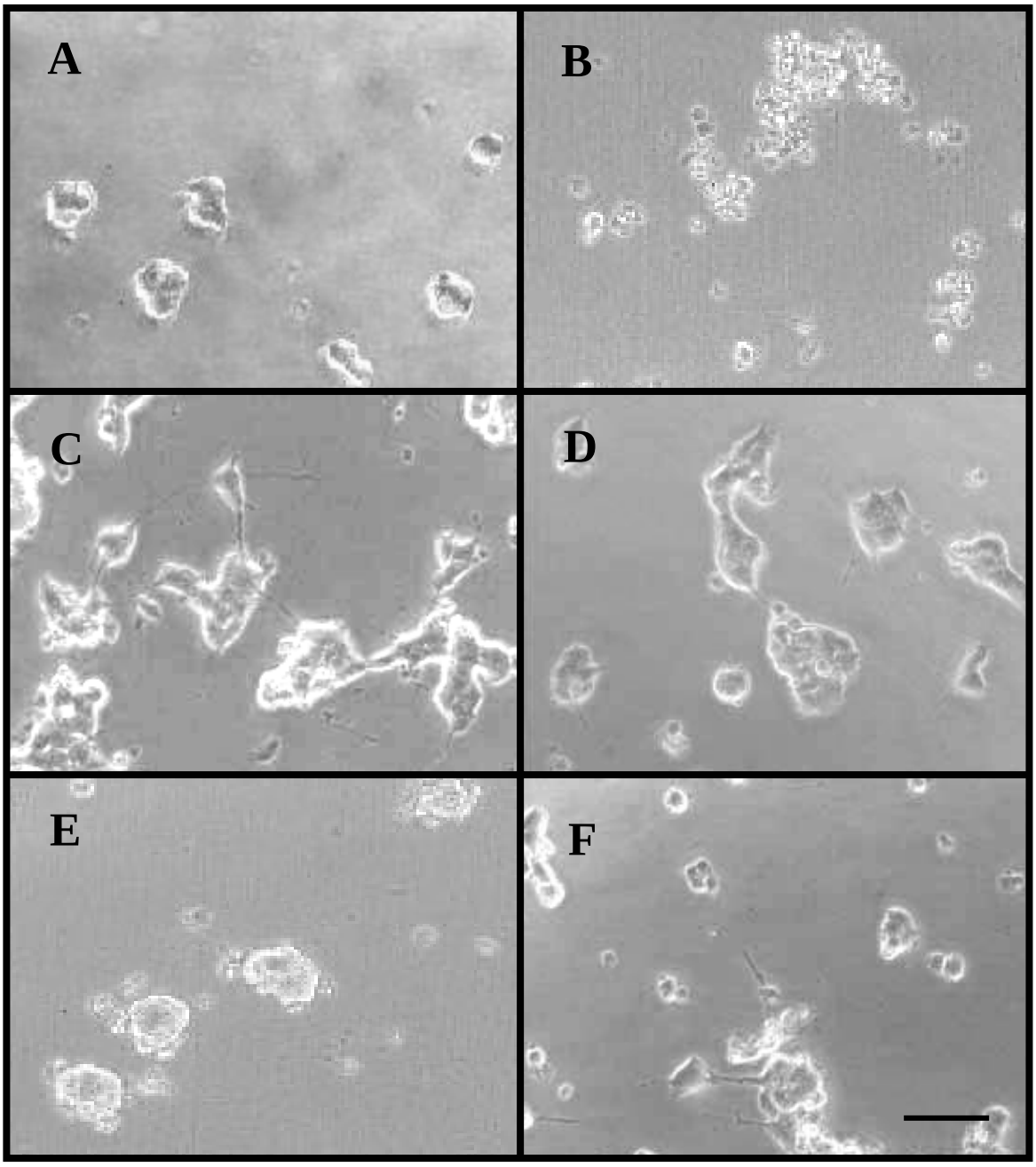


Fig. 6-2. Morphologies of neuronal PC12D cells after various treatment for 24 h. (A) Neuronal PC12D cells right before incubated in NGF-deprived serum free medium. Neuronal PC12D cells incubated in serum free medium in the (B) absence or presence of (C) 100 ng/ml NGF. (D) Neuronal PC12D cells treated with 1.5 μg/ml MC14 only. Neuronal PC12D cells treated with 0.4 ng/ml NGF in the absence (E) or presence (F) of 1.5 μg/ml MC14. Scale bar: 20 μM.

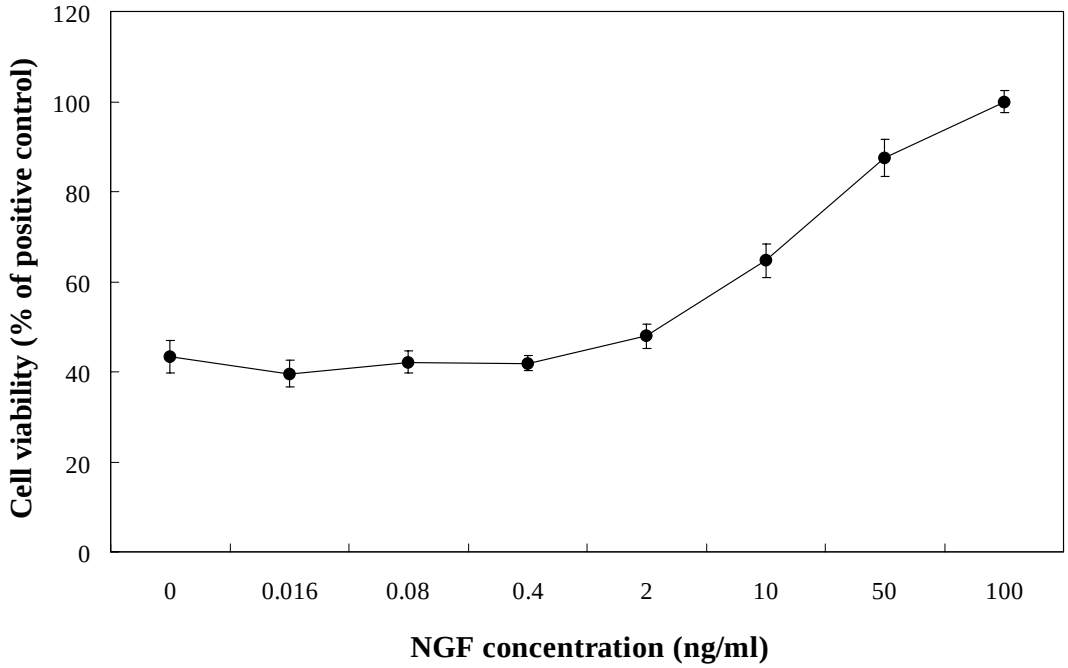


Fig. 6-3. Survival supporting effect of NGF on neuronal PC12D cells. Neuronal PC12D cells were incubated in the indicated concentrations of NGF in serum-free medium for 24 h. Cell survival was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4) .

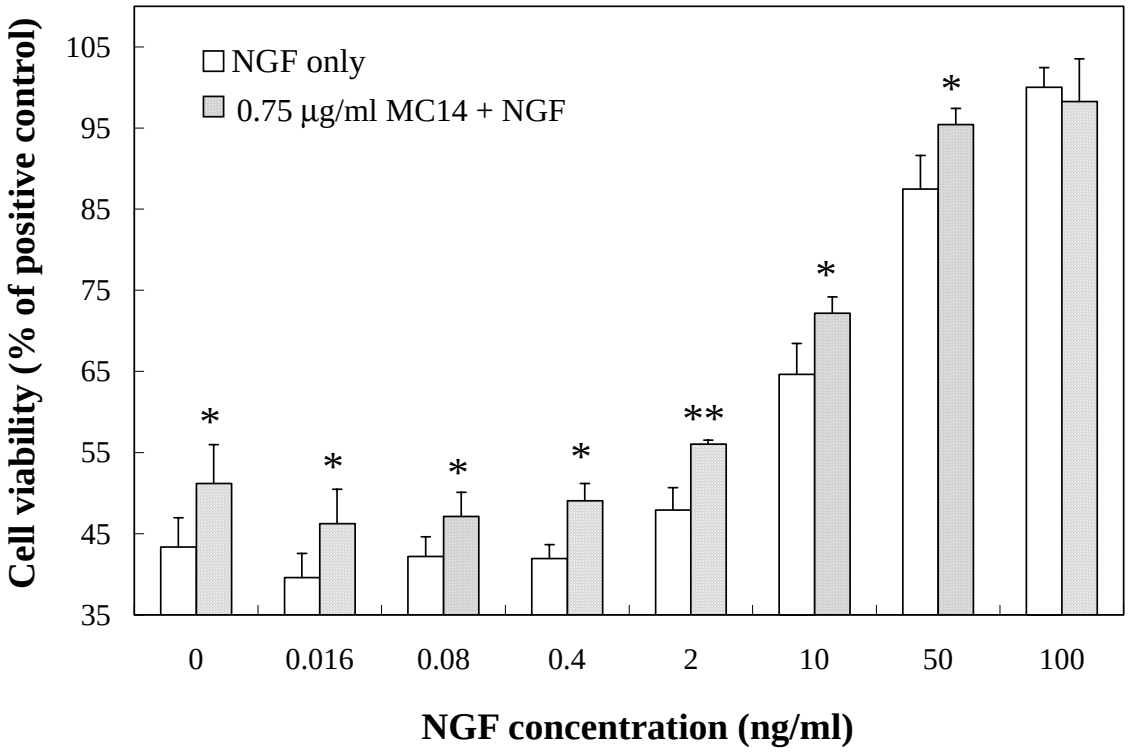


Fig. 6-4. Survival promoting effect of MC14 on neuronal PC12D cells. Neuronal PC12D cells were incubated in 0.75 μg/ml MC14 and the indicated concentrations of NGF in serum free medium for 24 h. Cell survival was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4). Significant difference from MC14-untreated control: ****P* < 0.05, *****P* **< 0.01 (Student's *t*-test).**

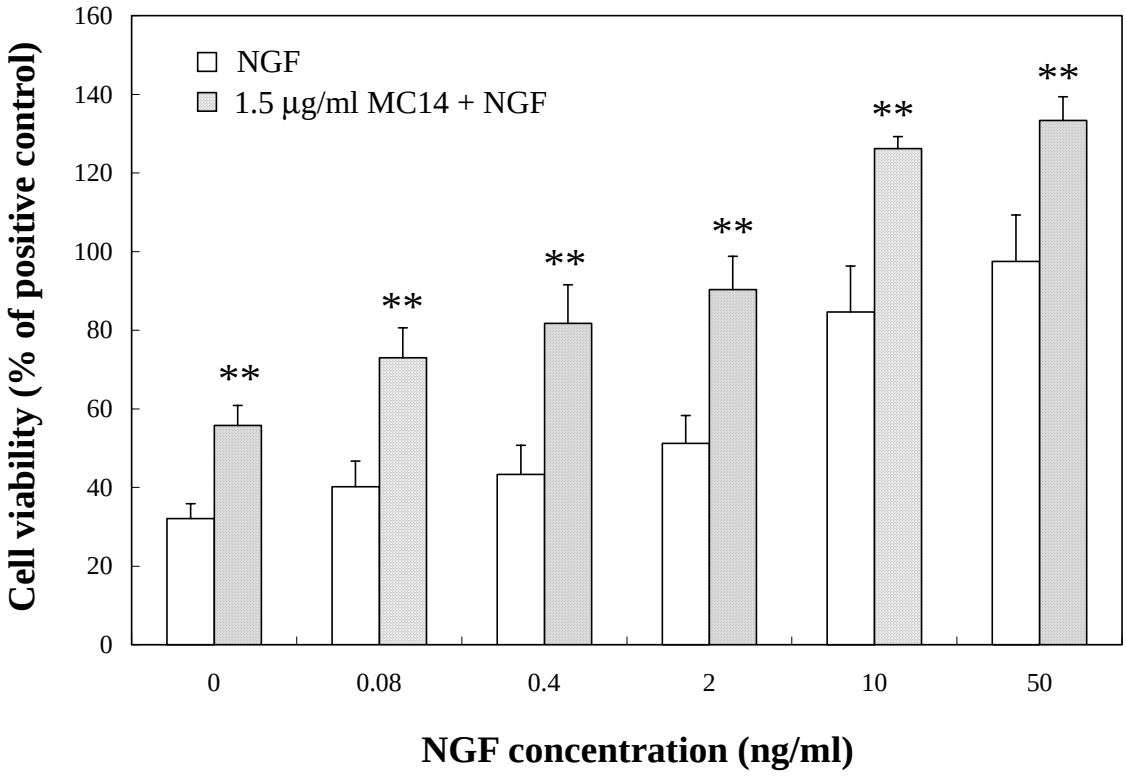


Fig. 6-5. Survival promoting effect of MC14 on neuronal PC12D cells. Neuronal PC12D cells were incubated in 1.5 μg/ml MC14 and the indicated concentrations of NGF in serum free medium for 24 h. Cell survival was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4). Significant difference from MC14-untreated control: **P < 0.01 (Student's *t*-test).

the combined effect of MC14 and 2 ng/ml NGF was comparable to positive control (Fig-6-5), demonstrating that the treatment of MC14 (1.5 μg/ml) can prevent serum-free-induced neuronal cell death even at a relatively low concentration of NGF (2 ng/ml). Besides, more than 40% of cells can be rescued from serum-free-induced cell death in an extremely low level of NGF (0.08-0.4 ng/ml). Another striking observation is that MC14 (0.75 and 1.5 μg/ml) could substantially support cell survival even in the absence of NGF (Figs. 6-2D, 6-4, 6-5), suggesting that MC14 possess significant neuroprotective effect, which was concentration-dependent (Fig. 6-6). Therefore, the mechanisms involved in survival supporting effect of MC14 and NGF under serum free medium were further investigated as described in the following section.

(4) Effect of various signaling molecule inhibitors on survival support activity of NGF in serum-free medium

Mounting evidence have shown that NGF-induced cellular responses are associated with the Ras-dependent stimulation of the mitogen-activated protein kinases and others have demonstrated that phosphatidylinositol 3-kinase may be important in survival of neuronal cells (Yao & Cooper, 1995; Suzukawa *et al*., 2000). To study the participation of these two signaling pathways in the NGF-induced survival support on neuronal PC12D cells under serum free condition, a specific MAPK kinase inhibitor PD98059, and a PKC inhibitor chelerythrine chloride were tested.

Pretreatment of cells with PD98059 did not reduce the percentage of cell survival induced by 0.08-10 ng/ml NGF (Fig. 6-7). Unexpectedly, the pretreatment of PD98059 significantly enhanced the survival suppoting effect induced by 10 ng/ml NGF.

With respect to the PKC inhibition, no significant inhibitory effect was detected by

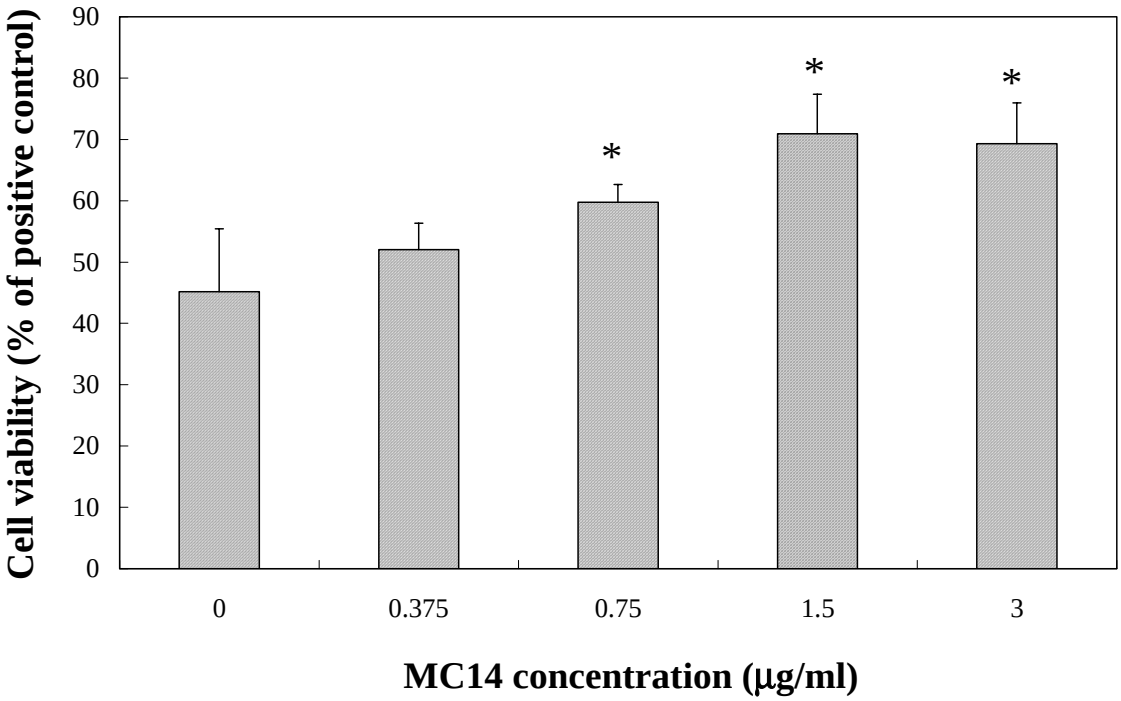


Fig. 6-6. Survival supporting effect of MC14 on neuronal PC12D cells. Neuronal PC12D cells were incubated in the indicated concentrations of MC14 in serum free medium for 24 h. Cell survival was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4). Significant difference from MC14-untreated control: *$P < 0.05$ (Student's *t*-test).

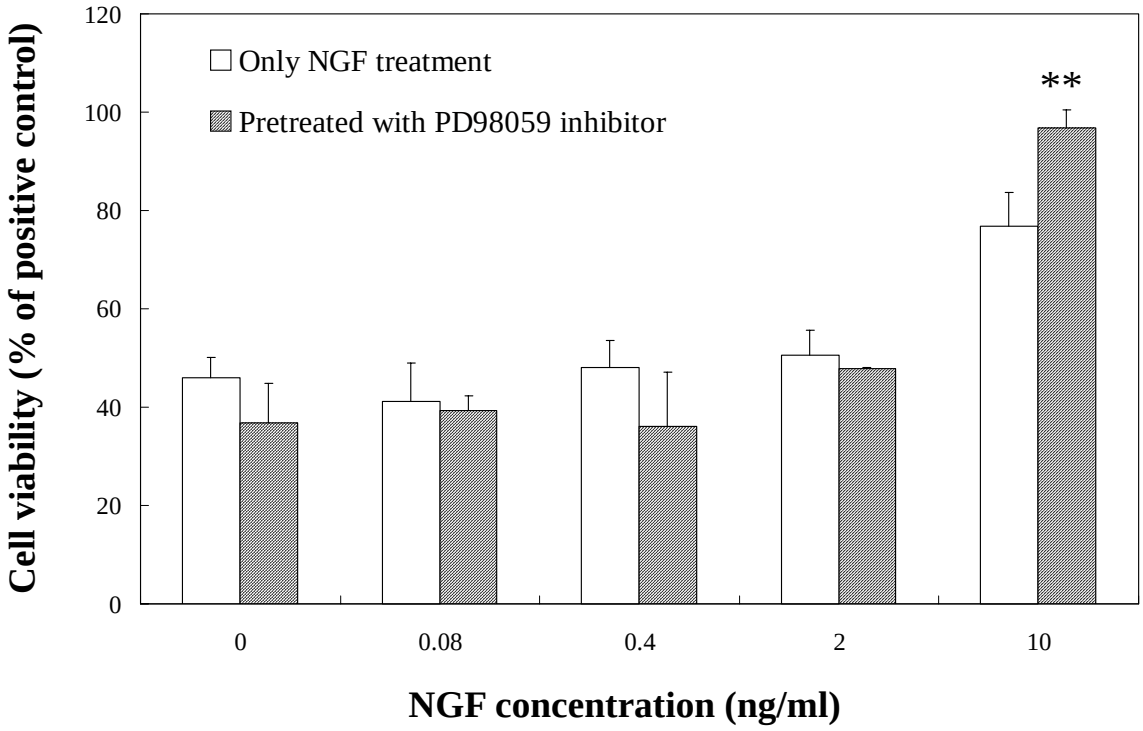


Fig. 6-7. Effect of PD98059 on survival supporting effect of NGF on neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without 3 μM PD98059 for 1 h before adding the indicated concentrations of NGF in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4). Significant difference from inhibitor-untreated control: **$P < 0.01$ (Student's *t*-test).

the pretreatment of cells with chelerythrine chloride in the presence of 0-10 ng/ml NGF (Fig. 6-8), suggesting that the NGF-rescuing effect on neuronal PC12D under serum-free condition may be independent of PKC-mediated pathway.

To confirm the effect of PD98059 and chelerythrine chloride, a dose higher than their effective concentration was examined on the neuronal PC12D cells treated with 10 ng/ml NGF. Result shows that chelerythrine chloride (600 nM and 6000 nM) did not significantly reduced the percentage of viable cell compared with inhibitor untreated control (Fig. 6-9). Again, significant enhancements of viable cell (more than 30%) were detected after the cells were pretreated with 3 and 10 μM PD98059 (Fig. 6-10). This indicates that the inhibition of MAP kinases activation may probably contribute to, rather than inhibit, the rescue effect of NGF on neuronal PC12D cells under serum free condition.

In order to further investigate the other potential signaling pathways that may mediate NGF-induced survival supporting activity on neuronal PC12D cells, PKA inhibitor and K252a were tested. Results indicated that no significant inhibitory effect of PKA inhibitor could be detected at 2, 10 and 50 nM (Fig. 6-11). However, a substantial reduction of cell viability was resulted after the treatment of 200 nM K252a (an effective inhibitory concentration for general protein kinases). Besides, a weak inhibition was observed at the treatment of 50 nM K252a while no significant change was detected in the cultures pretreated with 5 nM K252a (TrkA receptor inhibitory concentration) (Fig. 6-12). These results demonstrate that the neuronal survival supporting activity of NGF may not require the activation of PKA-mediated pathway and the activation of TrkA receptor while the activation of other intracellular kinases is essential for the NGF-mediated action.

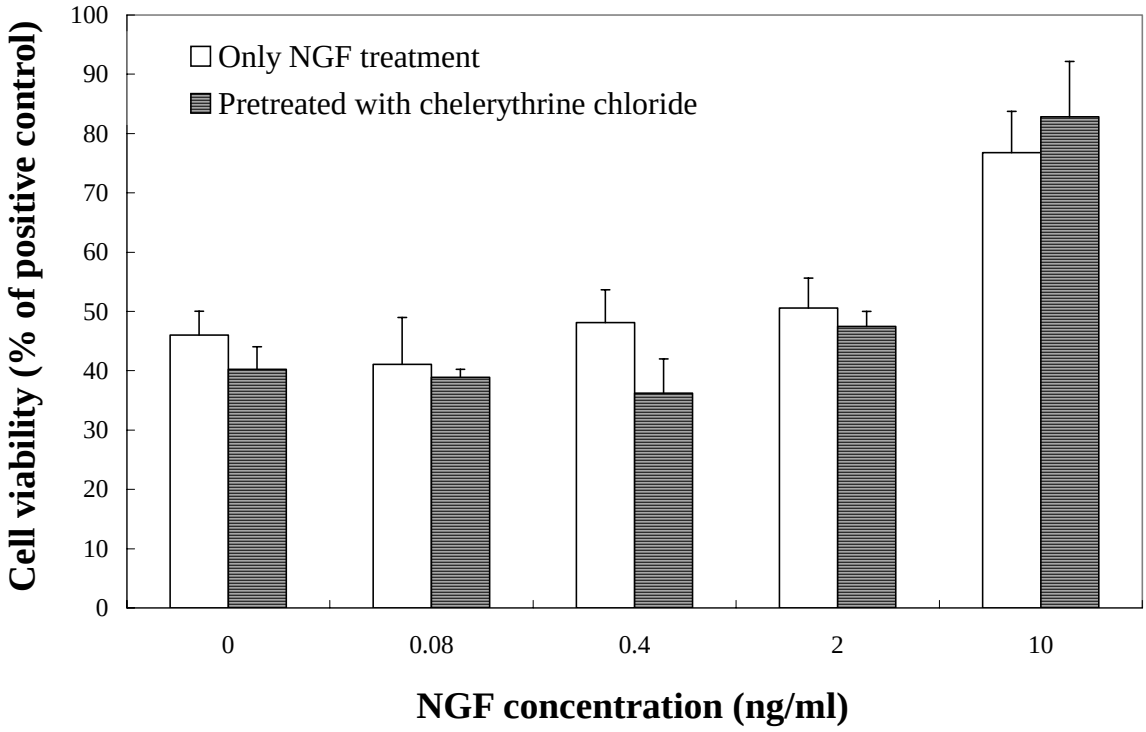


Fig. 6-8. Effect of chelerythrine chloride on survival supporting effect of NGF on neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without 600 nM chelerythrine chloride for 1 h before adding the indicated concentrations of NGF in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4).

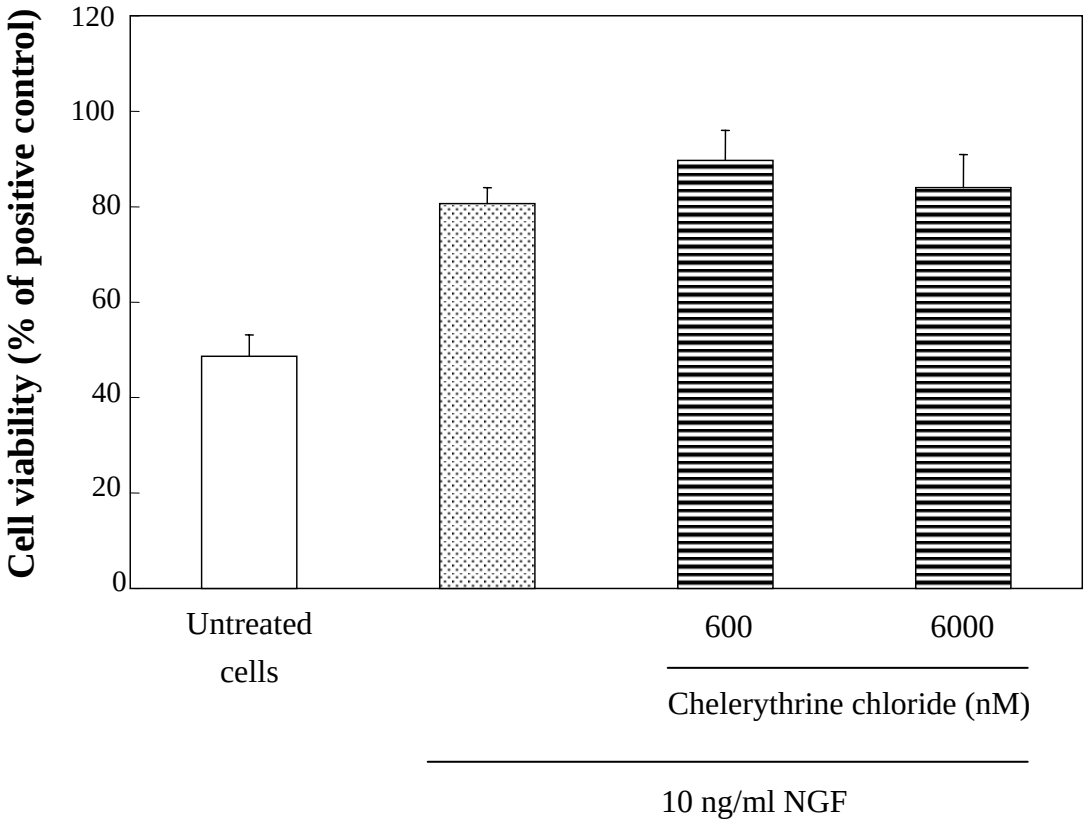


Fig. 6-9. Effect of chelerythrine chloride on survival supporting effect of NGF on neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without the indicated concentrations of chelerythrine chloride for 1 h before adding the 10 ng/ml NGF in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4).

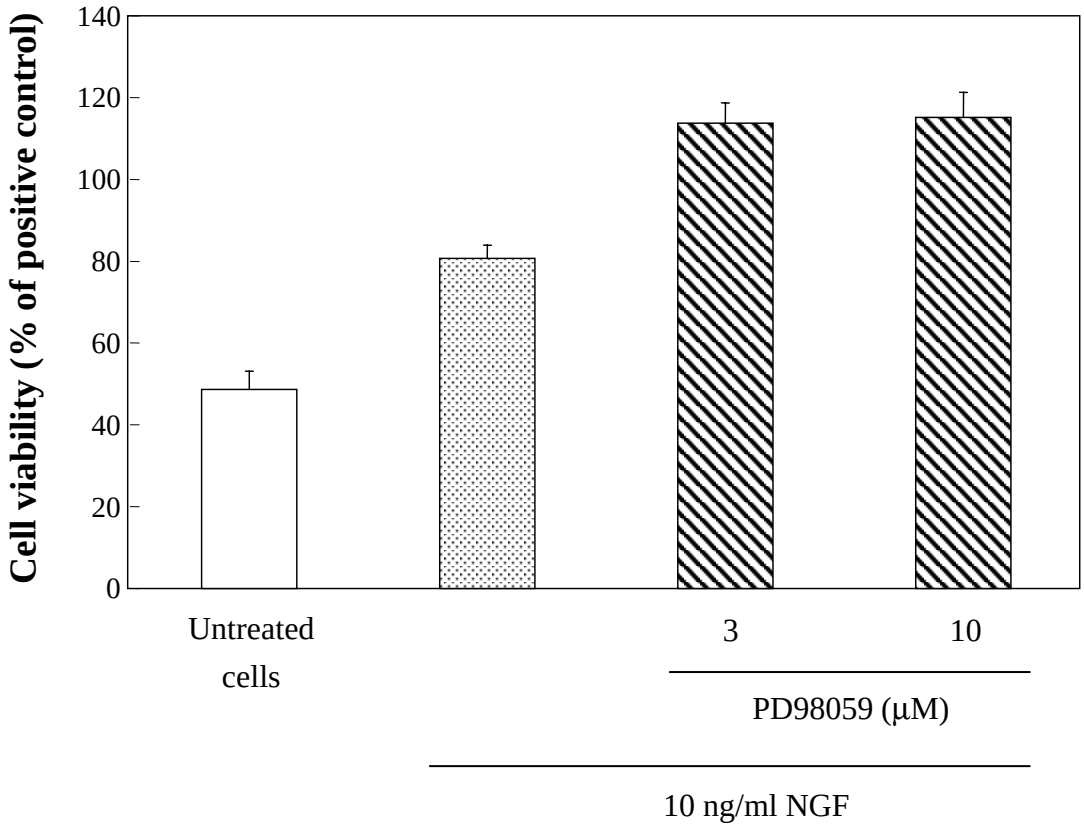


Fig. 6-10. Effect of PD98059 on survival supporting effect of NGF on neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without the indicated concentrations of PD98059 for 1 h before adding the 10 ng/ml NGF in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4).

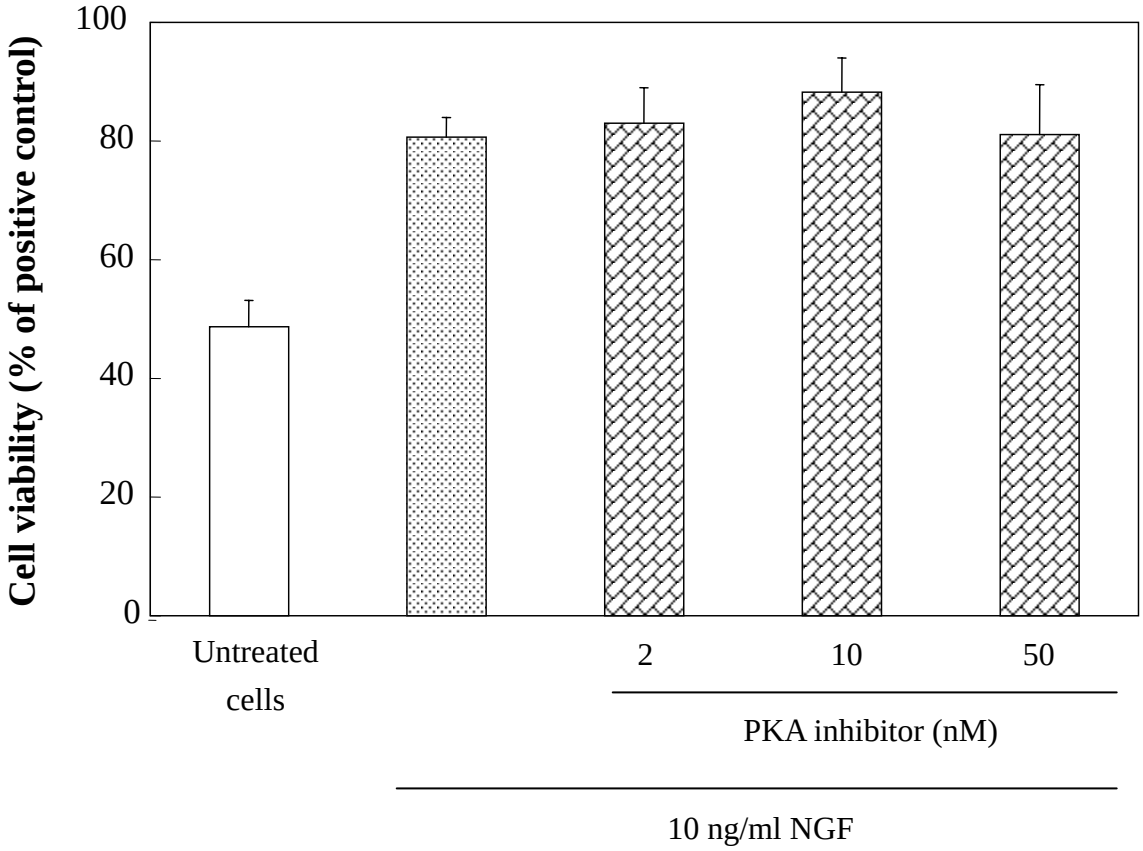


Fig. 6-11. Effect of PKA inhibitor on survival supporting effect of NGF on neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without the indicated concentrations of PKA inhibitor for 1 h before adding the 10 ng/ml NGF in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4).

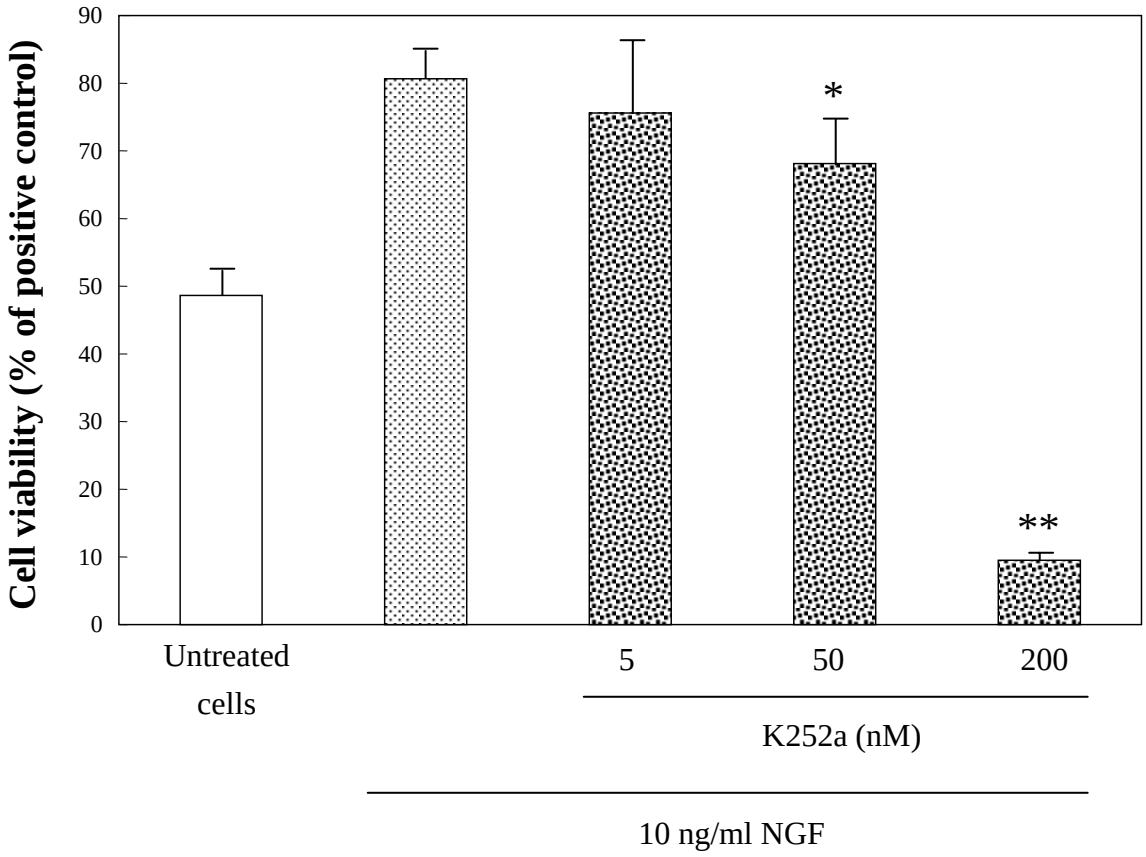


Fig. 6-12. Effect of K252a on survival supporting effect of NGF on neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without the indicated concentrations of K252a for 1 h before adding the 10 ng/ml NGF in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4). Significant difference from inhibitor-untreated control: *$P < 0.05$ **$P < 0.01$ (Student's *t*-test).

(5) Effect of signaling molecule inhibitors on survival supporting activity of MC 14

Next, the effect of various inhibitors on survival supporting effect of MC14 in the absence of NGF was studied. Pretreatment of neuronal PC12D cells with the test concentrations of K252a, chelerythrine chloride, PD98059 or PKA inhibitor showed no significant inhibition to MC14-induced cell survival effect on neuronal PC12D cells (Fig. 6-13), demonstrating that the activation of TrkA receptor, PKA, MAPK kinase or PKC were not required for the survival supporting effect of MC14.

6-1-3. Discussion

(1) Neuronal PC12D cell death induced by NGF-deprived serum-free medium

Neuronally differentiated PC12 cells have been considered to be the useful cell model for studying neuronal cell death because they exhibit the typical aspects, including the requirement of protein synthesis for the ‘programmed’ cell death, aggregation of chromatin, DNA fragmentation and conservation of mitochondria, by NGF withdrawal from serum free medium (Martin *et al*., 1988; Mesner *et al*., 1992). The present study describes the typical neuronal cell death of the neuronally differentiated PC12D cells, a subline of PC12 cells, can also be induced by NGF-withdrawal in serum-free medium. The aspect of time course of neuronal PC12D cell death resembles to that of sympathetic neurons and neuronal PC12 cells as reported previously (Mesner *et al*., 1992; Mesner *et al*., 1995). In addition, it has been reported that addition of anti-NGF antiserum caused the sympathetic neuron death between 24-

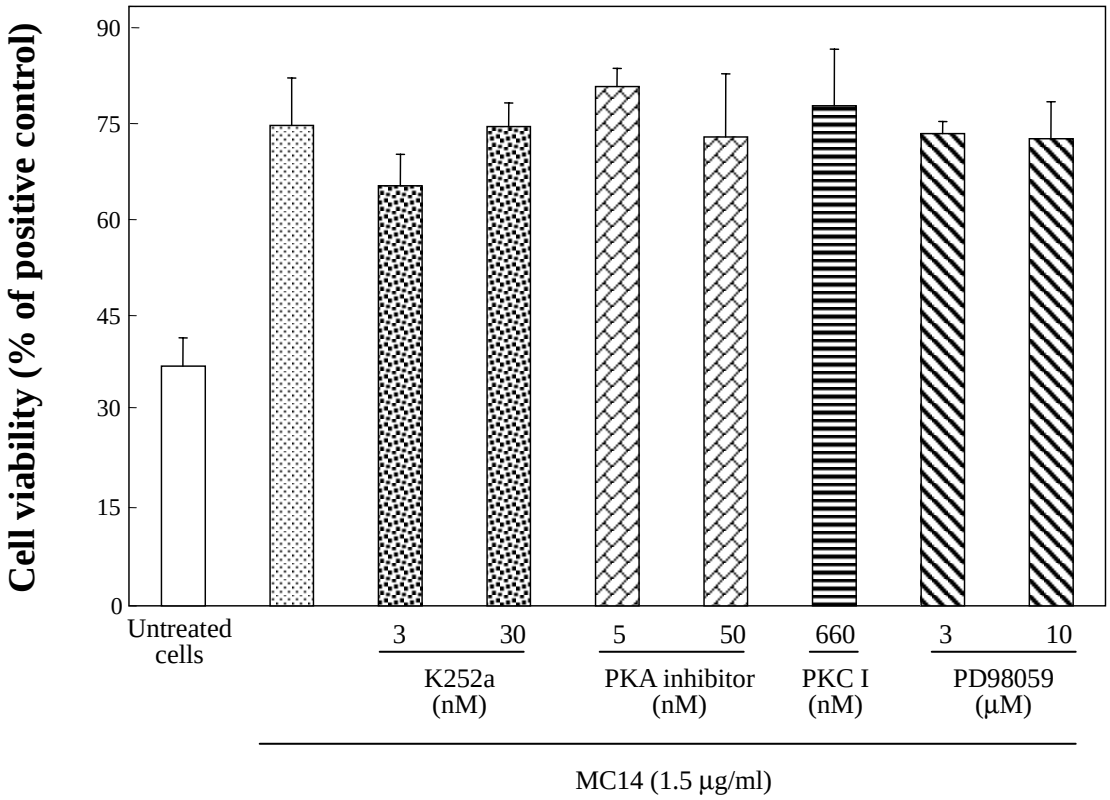


Fig. 6-13. Effect of various signaling molecular inhibitors on survival supporting effect of MC14. Neuronal PC12D cells were pretreated with or without the indicated concentrations of inhibitors for 1 h before adding 1.5 μg/ml MC14 in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4).

48 h by disruption of nuclear membrane, dilation of the rough endoplasmic reticulum and release of cytosolic enzyme (Martin *et al*., 1988). In consistent with these results, the present finding reveals that the metabolic activity of neuronal PC12D cells, as determined by MTT assay, significantly decreased within 24 h and almost completely inhibited after 48 h when the cells were incubated in NGF-deprived serum-free medium, suggesting that neuronal PC12D cells are also an ideal model system for the study of neuronal cell death by NGF-withdrawal under serum free condition.

(2) Survival supporting activity of NGF and MC14 on neuronal PC12D cells

NGF plays a vital role in regulating the normally occurring neuronal death during neurogenesis. NGF is secreted from the target cells and prevents the neuronal cell death at the time when these cells begin to be innervated (Barde, 1994). After the neuronal cells are fully differentiated, the maintenance of neuronal survival depends on the existence of NGF. If NGF is insufficient or deprived, neuronal cells will commit 'suicide' by eliciting a programmed cell death pathway (Semkova & Krieglstein, 1999). Death of the neuronal PC12 cells, like sympathetic neurons, can be prevented by NGF (10-100 ng/ml). Although the exact mechanism of NGF's survival-supporting effect remains to be determined, it has been known that certain gene expressions and cellular responses are induced by NGF to support cell survival (Bournat *et al*., 2000; Maggirwar *et al*., 2000). In this study, NGF is found to be capable of preventing the neuronal PC12D cell death in serum free medium, suggesting that they are responsive to NGF in terms of its survival supporting effect. More importantly, the neuronal PC12D cells can be used as a neuronal model system for the identification and verification of the biologically active substance that can support cell survival or promote NGF-induced

survival activity.

Results of the neuronal PC12D cell viability under the treatment of MC14 and NGF clearly indicate that MC14 can enhance NGF-induced cell survival activity. Treatment of cells with MC14 and a relatively low concentration of NGF (2 ng/ml) can prevent cells death as those treated with the optimal effective concentration of NGF (50 ng/ml). This result implies that age- or diseases-related neurodegeneration, which is induced by the deficiency of NGF in the brain region, might be alleviated or prevented by the treatment of MC14. In addition to effectively promoting NGF-induced cell survival, MC14 alone can significantly enhance the viability of neuronal PC12D cells in NGF-deprived serum free medium, demonstrating that it might trigger the similar signaling pathway employed by NGF for the survival support.

(3) Mechanisms of NGF-induced survival supporting effect on neuronal PC12D cells

The present study shows that the NGF-induced survival of neuronal PC12D cells is not affected by inhibiting the activity of TrkA receptor, suggesting that NGF support the survival of neuronal PC12D cells via the activation of signaling cascade other than that initiated by NGF-TrkA interaction. This result is in consistent with a study by Taglialatela *et al*.(1996), who showed that inhibition of TrkA phosphorylation does not abolish NGF rescue of serum-deprived PC12 cells from apoptosis. They used antisense oligonucleotide treatment to inhibit TrkA expression, and found that NGF can still rescue serum-deprived PC12 cells. Thus, NGF may rescue the TrkA-deficient PC12 cells by binding on the other growth factor receptor such as p75 receptor (Taglialatela *et al*., 1996). Therefore, it is speculated that NGF may bind to other surface receptor of the neuronal PC12D cells to elicit the TrkA-independent signaling pathway for the rescue

of cells under serum-free medium. However, it has also been reported that the activation of TrkA receptor is essential for the NGF-mediated survival in sympathetic neuron. (Belliveau *et al*., 1997) while the increase in the expression of TrkA receptor enhances the percentage of apoptotic neurons from chick embryo (Ahlemeyer *et al*., 2000). These conflicting results may be explained by the different type of neurons in the experiments. The present study showed that NGF-induced cell survival is mediated via the intracellular kinase activity as substantial cell death was observed under the treatment of 200 nM K252a, at which the intracellular kinases are inhibited nonspecifically.

Concerning the effects of chelerythrine chloride on NGF-induced rescue on the neuronal PC12D cells, it shows no inhibitory effect on NGF-induced survival support, demonstrating that PKC- mediating signaling pathways may not involve in this action of NGF. Interestingly, it has been reported that phosphatidyl-inositol-3 (IP3) kinase is required in the prevention of apoptosis by NGF (Yao & Cooper, 1995). As protein kinase C is a downstream effector protein kinase in the IP3-signaling cascade, NGF may activate IP3, which subsequently activates the other immediate effector protein kinase as a divergent pathway. Thus, NGF supports survival of neuronal PC12D cells even the activity of PKC is blocked. Alternatively, neuronal PC12D cells might not express enough IP3 level in serum-free medium and so the IP3-dependent signaling cascade is not activated by NGF at all.

On the other hand, PKA is apparently independent of NGF-induced survival supporting effect on neuronal PC12D cells as no inhibitory effect is observed for the PKA inhibitor even at its 10-time PKA inhibiting concentration (50 nM). This result clearly demonstrates that PKA is not required for NGF rescue of neuronal PC12D cells. This is consistent with that reported by Rukenstein *et al*., they used a variant PC12 cells

with defective PKA activity to show that NGF, cAMP, insuline and FGF could still promot long-term survival of this variant PC12 cells in serum free medium. This result strongly suggests that the activation of PKA is not required for the survival actions of these growth factor.

Although the Ras-MAP kinase pathway has been considered to be actively involved in neuronal differentiation of PC12 cells induced by NGF (Traverse *et al*., 1992; Pang *et al*., 1995; Hashimoto *et al*., 2000), its role during the NGF-induced survival support is less described. The present study using the specific MAPK kinase inhibitor PD98059 indicates that the inhibition of MAP kinases activity does not block the survival supporting effect of NGF, but rather enhances the cell viability in serum free medium compared with the effect of inhibitor-untreated control (Figs 6-7,6-10). In other words, the inactivation of MAP kinases promotes the survival supporting action of NGF in neuronal PC12D cells in serum free condition. This implies that MAP kinase-mediated pathways might involve in cell death signal, instead of survival supporting signal in neuronal PC12D cells incubated in serum free medium. It has been demonstrated that another MAP kinase inhibitor olomoucine is able to prevent apoptosis of central and peripheral neurons by inhibiting JNK (c-Jun N-terminal kinases) and its activation leads to neuronal apoptosis (Maas *et al*., 1998). Despite the mechanism of inhibitory action of olomoucine and PD98059 on MAP kinase may be different, MAP kinase inhibited by PD98059 in the neuronal PC12D cells may involve in NGF-deprived induced apoptotic cell death.

(4) Mechanisms of survival supporting activity of MC14 on neuronal PC12D cells

To investigate the signal transduction pathways mediating the survival supporting

effect of MC14, the inhibitory effects on several typical signaling pathways were examined. Pharmacological inhibition of TrkA, PKC, PKA or MAP kinase results in no significant inhibition of MC14-enhanced survival support on neuronal PC12D cells under serum-free and NGF-deprived conditions. This result demonstrates that MC14 rescues neuronal PC12D cells via a novel signaling pathway independent of PKC, PKA and MAP kinase. In addition, MC14 does not directly bind on and activate the NGF-specific tyrosine receptor kinase A. Recently, accumulative evidence have shown that the activation of transcription factor NF-κB (nuclear factor-κB) in neuronally differentiated PC12 cells and sympathetic neurons can prevent NGF-deprived cell death and other apoptotic cell death (Maggirwar *et al*., 1998; Foehr *et al*., 2000; Maggirwar *et al*., 2000). In addition, a number of recent reports have indicated that PI3 kinase (phosphatidylinositol 3-kinase) and a major substrate of PI3 kinase activity, Akt/PKB, regulates the survival of several cell types (Franke *et al*., 1997; Crowder & Freeman, 1998). Additionally, constitutive activation of PI3 kinase or Akt/PKB contributes to NGF-independent survival functions in sympathetic neurons (Crowder & Freeman, 1998). It is speculated that MC14 might support the survival of neuronal PC12D by activating the PI3-Akt/PKB pathway, subsequently activating the NF-κB to protect cells in NGF-deprived serum free medium. Taken together, the present result demonstrates that MC14 exhibits a NGF-independent survival function to rescue the NGF-deprived neuronal PC12D cell. Additionally, MC14 can also complement the survival supporting effect of NGF to rescue neuronal PC12D cells in serum free medium.

(5) Neurite outgrowth promoting effect and survival supporting effect of MC14 are regulated via different mechanisms in PC12D cells

Comparing the findings described in this chapter and chapter 4, the mechanisms of actions of MC14 for neuronal differentiation and survival support are regulated by distinct signaling pathways in PC12D cells. Exposure of naïve PC12D cells to NGF induces differentiation and neurite outgrowth from PC12D cells. The neurite-outgrowth promoting mechanisms of MC14 involve the facilitation of NGF binding to its receptor TrkA, and the activations of PKA and MAPK kinase. In addition, the promoting effect of MC14 is mediated by at least two separate signaling pathways, namely TrkA-MAPK pathway and PKA-MAPK pathway. They probably complement each other to promote the action of NGF for neurite outgrowth and neurodifferentiation of PC12D cells. Regarding the survival supporting effect of MC14, TrkA receptor is apparently not involved in the action of MC14. This may be explained by the incapability of MC14 to directly bind on the receptor TrkA. In contrast to neurite outgrowth promoting activity of MC14, the PKA-mediated cascade is also not required for MC14 to rescue neuronal PC12D cells in NGF-deprived serum free medium. An interesting finding in these studies is that although MAP kinase-mediated pathway has a key role in controlling neuronal differentiation of PC12D cells, this pathway seems to have reverse action on survival support as the blockage of the MAP kinase-pathway by PD98059 results in enhancing the NGF-induced survival support effect on neuronal. On the other hand, several different properties of MC14 on PC12D cells can also be found in the present studies. The most striking finding is that MC14 alone can support survival of neuronal PC12D cells in NGF-deprived serum free medium, but MC14 fails to induce neurite outgrowth from PC12D cells in the absence of NGF.

Three important implications from these findings can be derived. First, the treatment of MC14 may not only potentiate the action of NGF for neuronal

differentiation during the developmental stage of mammals, but also rescue the apoptotic cell death caused by NGF-unrelated (e.g. Parkinson's disease) or NGF-related (e.g. Alzheimer's disease) neurodegenerative disorders, as well as naturally occurring neurodegeneration in the CNS. Second, MC14 activates distinct intracellular signaling pathways to bring about different cellular responses such as neurodifferentiation and maintenance of survival. The critical factors determining these divergent actions of MC14 include the developmental state of PC12D cells (i.e. naïve PC12D cells vs neuronally differentiated PC12D) and the culture environment (i.e. serum containing vs serum-free medium). Third, the bioactive substance showing survival supporting activity is not necessarily capable of inducing neurodiferentiation. Apart from MC14, several substances have been reported to have neuron-rescuing activity but fail to induce neurite outgrowth (Rukenstein *et al*., 1991; Belliveau *et al*., 1997; Ashcroft *et al*., 1999; Singer *et al*., 1999). Hence, it should be cautious when screening the samples for neuroprotective substances using the neurite outgrowth inducting bioassay on PC12D cells.

In conclusion, the present study provide direct evidence to show that MC14 can support the neuronal survival, and promote the rescuing effect of NGF under serum-free medium. This demonstrates that MC14 may be a promising therapeutic agent to treat neurodegenerative disorders such as Alzheimer's diseases.

6-2. Survival supporting effect of NGF and MC14 against oxidative stress on PC12D cells

Oxidative stress has been implicated in the pathophysiology of many

neurodegenerative diseases including Parkinson's disease (PD) and Alzheimer's disease (AD) (Schulz *et al*., 2000; Gilgun-Sherki *et al*., 2001). Oxidative stress can cause cellular damage and subsequent cell death mainly by apoptosis as the reactive oxygen species oxidize vital cellular components such as lipids, protein and DNA (Gorman, 1996; Simonia & Coyle, 1996). In fact, the brain is exposed throughout life to excitatory amino acids whose metabolism produces reactive oxygen species, thereby promoting excitotoxicity (Gilgun-Sherki *et al*., 2001). Beta-amyloid over-production represents another form of oxidative stress. Pappolla *et al* (1998) provided evidence for the hypothesis that beta-amyloid, the product of abnormal processing of the amyloid protein precursor (APP) in AD, is neurotoxic and that such toxicity is mediated by free radicals in vitro and in transgenic mice model of AD. These findings suggest that the oxidative stress resulted from the over-production of beta-amyloid is likely to be one of the pathologies leading to Alzheimer's disease.

The most common cellular free radicals are hydroxyl radical ($OH^{\bullet}$), superoxide radical ($O_2^{-\bullet}$), and nitric oxide ($NO^{\bullet}$) (Jenner and Olnaw, 1996; Simonia and Coyle, 1996). Other molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrate (ONOO), are not free radicals, but can lead to their generation through ferrous iron-mediated chemical reactions. Free radicals and related molecules are often classified together as reactive oxygen species (ROS) to signify their ability to promote oxidative changes within the cell (Simonia and Coyle, 1996). The follow chemicals reactions demonstrate the processes of free radical formation inside the cell (Gilgun-Sherki *et al*., 2001).

$$\text{Substrate} + O_2 \xrightarrow{\textit{Mixed function oxidase}} \text{Product} + H_2O_2$$

$H_2O_2 + Fe^{2+} \longrightarrow OH^- + {}^{\bullet}OH + Fe^{3+}$

Therefore, the treatment of cells with hydrogen peroxide can mimic the intracellular oxidative stress (Jackson *et al*., 1990; Xiao *et al*., 2000). In the present study, hydrogen peroxide-induced cell death of PC12D was used as an in vitro study to investigate the neuroprotective effect of MC14 against H_2O_2-insult. The result would provide important pharmacological evidence for the potential of MC14 as a therapeutic agent for the treatment of neurodegenerative diseases such as AD and PD.

6-2-1. Methods and Materials

(1) Bioassay of H_2O_2-induced cell death and neuroprotective activity of NGF and MC14

PC12D cells were maintained as described in section 6.2.1. For bioassay, cells were seeded on poly-L-lysine coated 96-well plate at a density of $5x10^4$ cells per well in complete medium. Experiments were carried out after 24 h incubation at 37°C in a humidified atmosphere containing 5% CO_2. Hydrogen peroxide (H_2O_2) was freshly prepared from 30% stock solution prior to each experiment to produce oxidative stress. The cells were preincubated with NGF and/or MC14 for 2 h before the addition of H_2O_2. Cell viability was determined by MTT assay after incubating the cells in H_2O_2 for 6 h.

(2) Extraction and electrophoretic analysis of DNA

Soluble DNA in PC12D cells was extracted by the method of Hockenbery *et al*.

(1990) with minor modifications. In brief, PC12D cells ($5x10^6$ cells) were seeded on 10-cm culture dish in complete medium. After 24 h, aliquot of freshly diluted solution of H_2O_2 was added to the dish to make a final concentration of 400 μM. Control cells were treated in complete medium only. After incubation for 12 h, cells were harvested by trituation and washed twice with ice-cold PBS. Cell pellets were collected by centrifugation at 1000 rpm for 10 min. Then, cells were lysed with 300 μl lysis buffer (0.5% Triton X-100, 5 mM Tris buffer at pH 7.4 and 20 mM EDTA) for 30 min at 4 °C. The supernatants were collected after centrifugation at 27,000 g for 15 min. Soluble DNA in the supernatant fraction of the cell lysate was extracted sequentially with an equal volume of phenol, phenol-chloroform and chloroform. RNase was added to the samples to a final concentration of 20 μg/ml, and the solutions were incubated at 37°C for 60 min. The samples were further extracted twice with phenol-chloroform. Subsequently, DNA was precipitated with 1/10 sample volume of 3 M sodium acetate (pH 5.2) and two volumes of ethanol at -80°C overnight. DNA pellets were collected by centrifugation at 13,000 rpm for 30 min and washed with 70% ethanol. DNA pellets were dried and redissolved in 20 μl sterilized water. DNA concentration was determined by UV absorbance at 260 nm. Three μg of DNA per sample were subjected to electrophoresis on a 3.5% polyacrylamide gel. The markers DNA λ*Hind* III and 100 bp DNA were used to determine the size of DNA.

6-2-2. Results

(1) Induction of cell death by H_2O_2

The H_2O_2-induced PC12D cell death was determined by MTT assay and checked by

microscopic observation. In the experiments, cell culture incubated in complete medium without H_2O_2 treatment was used as a control and assigned as 100% cell viability. As shown in Fig. 6-14, H_2O_2-induced cell death exhibited a concentration dependent manner. Cell viability was slightly but significantly decreased after PC12D cultures were exposed to 200 μM H_2O_2 whereas a substantial reduction of viable cells (48%) was detected when the cells were exposed to 400 μM H_2O_2. At 800 μM H_2O_2, the cell viability further dropped to 23% compared with that of untreated control. Under microscopic observation, the number of shrunk cells increased with H_2O_2 concentration (Photo not shown) and it confirmed the data obtained from viability determination by MTT assay. Moreover, the dead cells were shrunk into a dense dark particle with intact plasma membrane (observed by microscopy, photo not shown), indicating that H_2O_2 induced the typical apoptotic cell death in PC12D cells (Mesner *et al*., 1992). To confirm whether H_2O_2 induced PC12D cell death via apoptosis, soluble DNA of PC12D cell was isolated after H_2O_2-treatment and then analysed by polyacrylamide gel electrophoresis. As shown in Fig. 6-15 (lank 4), the soluble DNA fraction contained ladder-like pattern of DNA fragments with molecular weight of approximately a multiples of 180 base pairs, which is equivalent to the length of nucleosome subunit after DNA cleavage by nuclease, after the cells were treated with 400 μM H_2O_2 for 24 h. Whereas no fragmented DNA ladder was observed in the untreated control cells (Fig. 6-15, lank 3), suggesting that H_2O_2 induced apoptotic death in PC12D cells.

(2) Protective activity of NGF against H_2O_2-induced apoptosis

Protective effect of NGF against H_2O_2-induced apoptosis in PC12D cells was shown in Fig. 6-16. A concentration-dependent neuroprotective effect of NGF was observed in

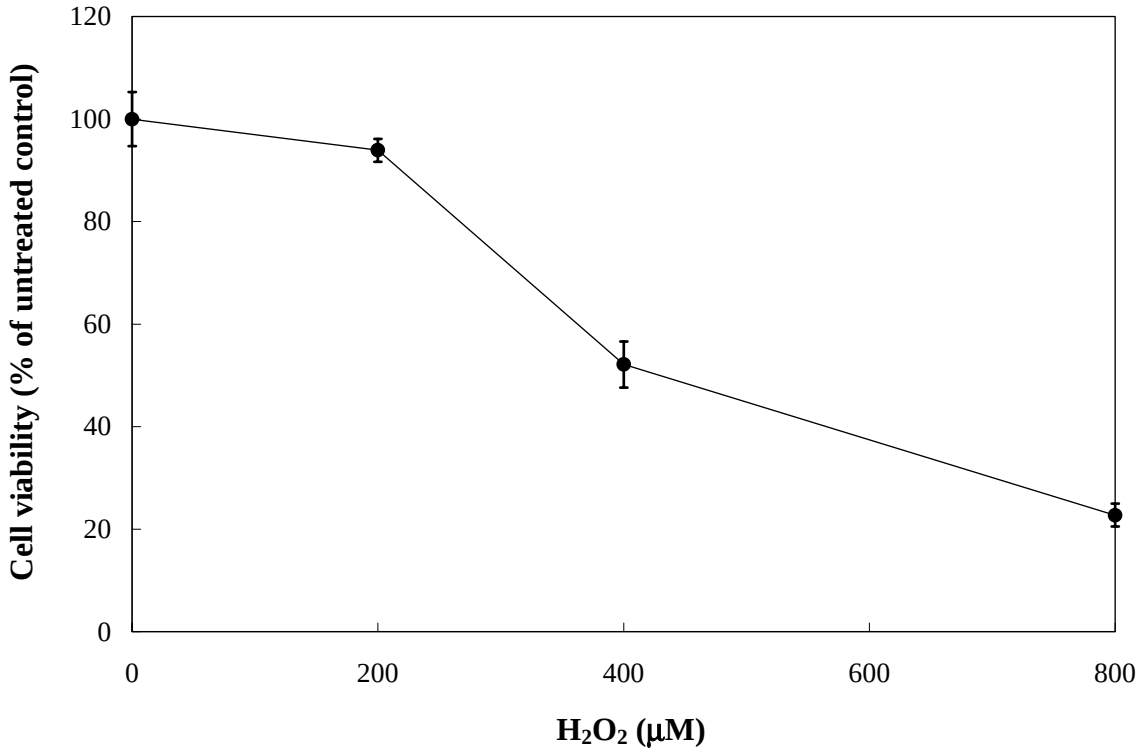


Fig. 6-14. Hydrogen peroxide-induced PC12D cell death. PC12D cells were treated with the indicated concentration of H_2O_2 for 6 h. Cell viability was determined by MTT assay, and expressed as a percentage relative to the untreated control (complete medium, 100%). Each point represents the mean ± SD (n=4).

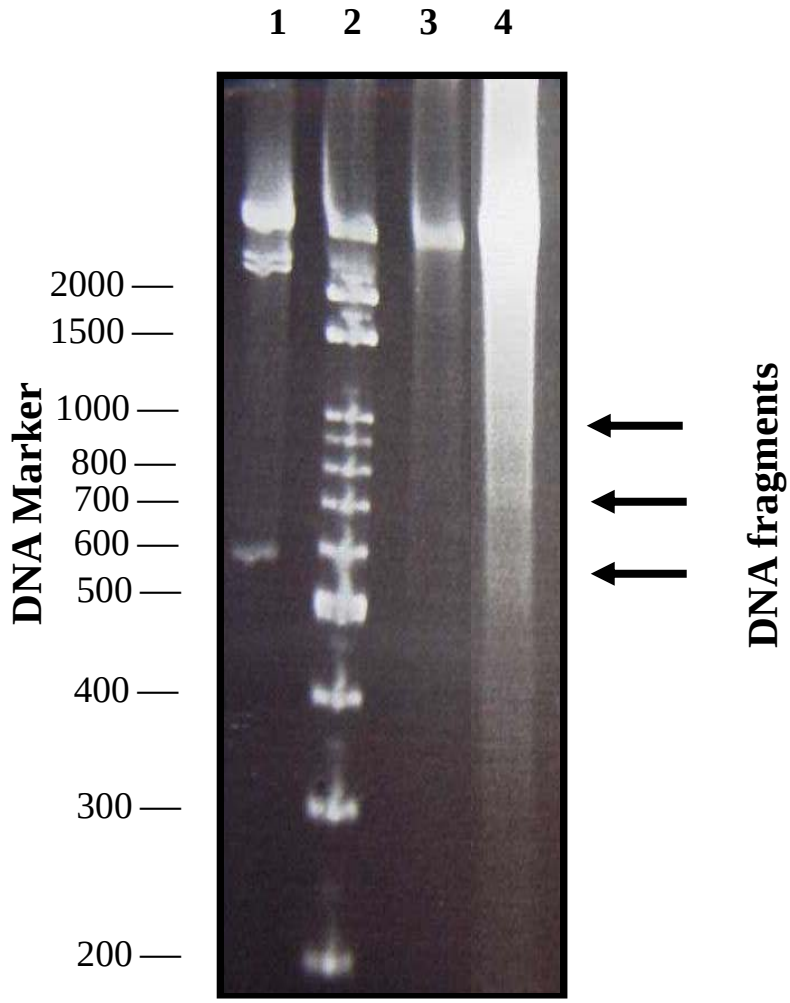


Fig. 6-15. Polyacrylamide gel eletrophoretic analysis of soluble DNA of PC12D cells after H_2O_2-insult. PC12D cells were incubated with or without 400 μM H_2O_2 for 24 h. Soluble DNA was extracted and electrophoresed on 3.5% polyacrylamide gel. Lane 1: DNA λ*Hind* III markers. Lane 2: 100 bp DNA ladder markers. Lane 3: Untreated cells in complete medium. Lane 4: Cells treated in 400 μM H_2O_2 for 24 h. Arrows indicate DNA fragments in PC12D cells induced by H_2O_2 insult.

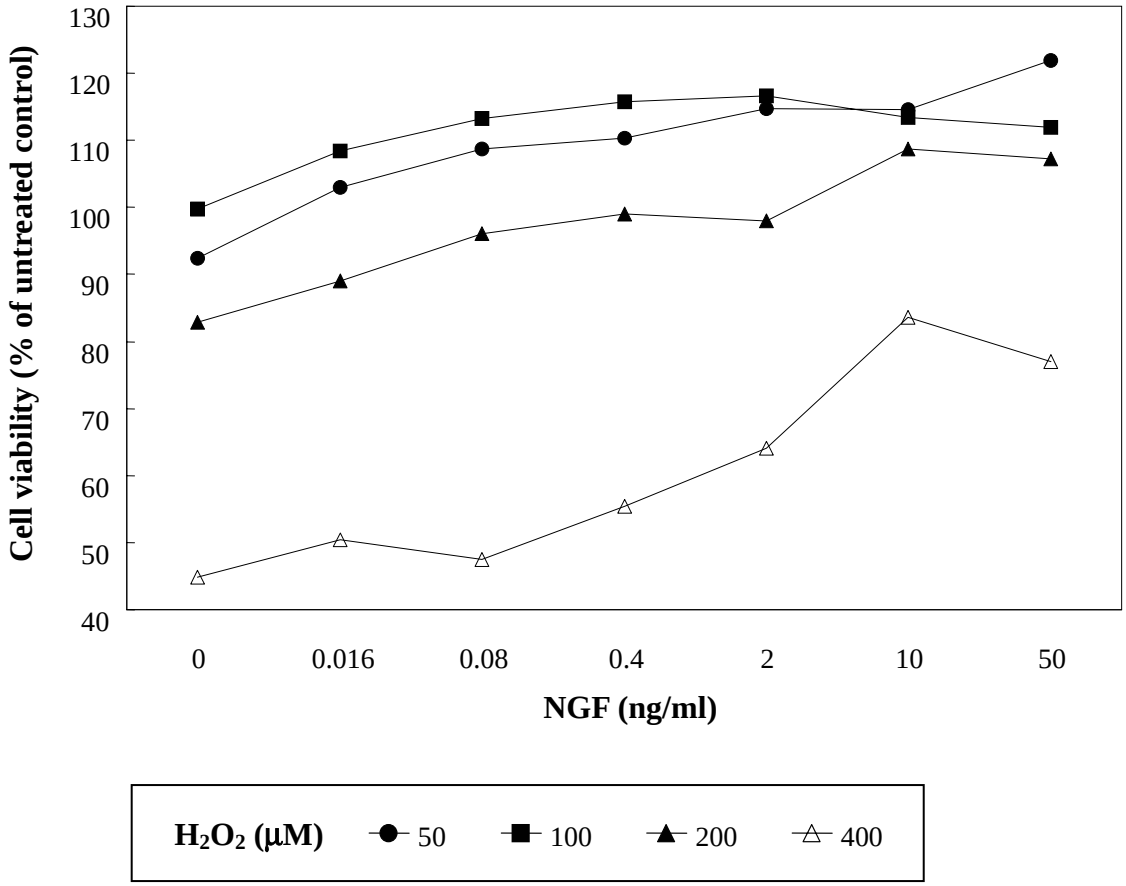


Fig. 6-16. NGF-induced neuroprotective effect against H_2O_2-insult on PC12D cells. PC12D cells were pretreated with the indicated concentration of NGF for 2 h and subsequently treated with the indicated concentration of H_2O_2 for 6 h. Cell viability was determined by MTT assay, and expressed as a percentage relative to the untreated control (complete medium, 100%). Each point represents mean value (n=3).

the cultures exposed to 50-400 μM H_2O_2, suggesting that NGF can induce protective mechanisms against the H_2O_2-insult on PC12D cells.

(3) Survival promoting effect of MC14 against H_2O_2-induced apoptosis

When the cells were preincubated with 3 μg/ml MC14 and NGF (0.4-50 ng/ml) for 2 h, H_2O_2-induced cell injury was significantly attenuated compared with those treated with NGF alone. Results of MTT assay show that 27-47% of cell viability were enhanced by MC14, suggesting that MC14 could significantly promote NGF-induced survival activity against the oxidative insult on PC12D cell. In addition, it should be noted that the preincubation of MC14, even in the absence of NGF, could enhance cell viability by 2.7-fold compared with the MC14-untreated control (Fig. 6-17). This result clearly demonstrates that MC14 can significantly promote cell survival in the presence of NGF, and protects PC12D cells against H_2O_2-induced apoptosis.

6-2-3. Discussion

There are substantial evidence showing that many neurodegenerative diseases such as AD and PD are brought about by the induction of highly intracellular regulated program (also known as apoptosis) that leads to neuronal death (Cotman 1998; Gilgun-sherki *et al*., 2001). Loss of trophic support, DNA damage, excitotoxic and beta-amyloid insults are believed to be the main cause of such death. Since markers of DNA damage and apoptosis have been shown to be present in the AD brain even at the early stage of the disease, if left the apoptotic damage unrepaired, the brain will become more vulnerable to further pathology (Xiao *et al*., 2000). An in vitro study has shown that

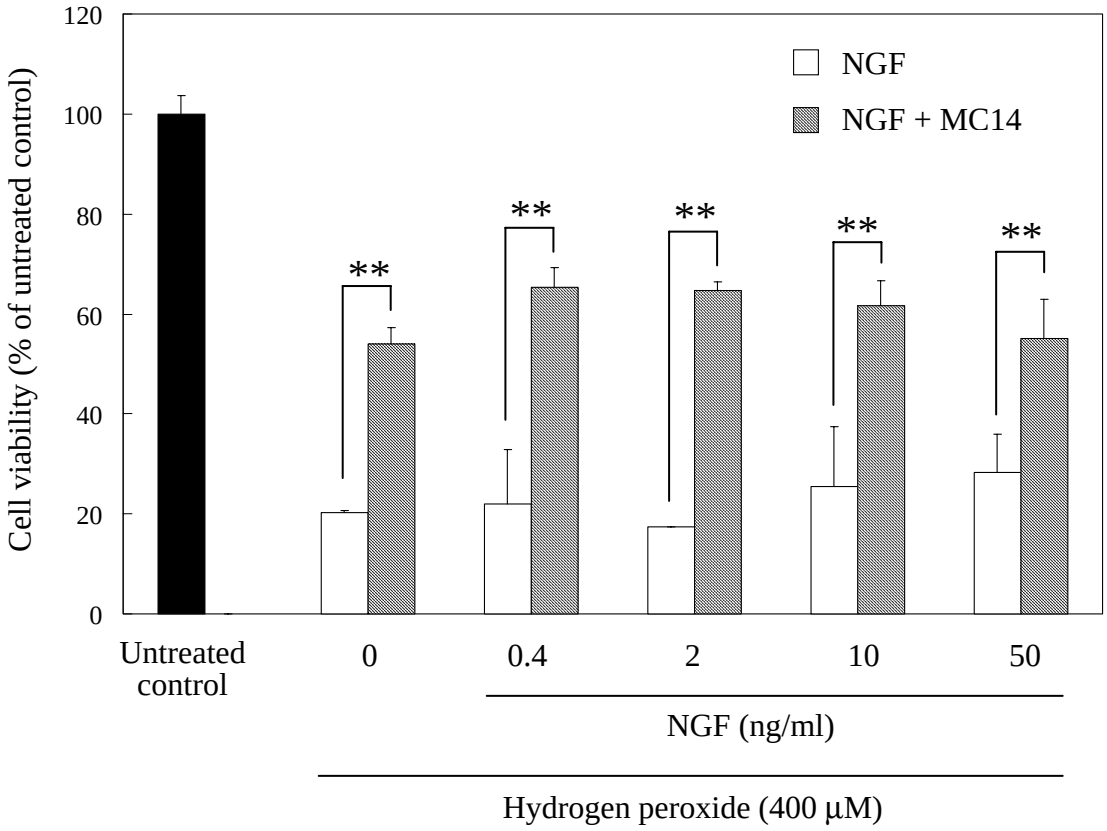


Fig. 6-17. Neuroprotective promoting effect of MC14 against H_2O_2-insult on PC12D cells. PC12D cells were pretreated with 3 μg/ml MC14 and the indicated concentration of NGF for 2 h before the addition of 400 μM H_2O_2. After 6 h incubation, cell viability was determined by MTT assay, and expressed as a percentage relative to the untreated control (complete medium, 100%). Each point represents mean value ± SD (n=4). Significant difference from the corresponding NGF-only control: **$P < 0.01$ (Student's *t*-test).

treatments that decrease the oxidative stress protect neurons following beta-amyloid insult (Behl *et al*., 1999). This result indicates that oxidative stress is the key mediator in apoptotic paradigms. Therefore, therapeutics addressing to this fundamental mechanism might not only slow down the degenerative process, but also restore or promote the neuronal function. As the major component of reactive oxygen species, H_2O_2 has been extensively used as an inducer of oxidative stress in PC12 cells (Jackson *et al*., 1990; Satoh *et al*., 1996; Lee *et al*., 2000). The present study confirmed that H_2O_2 also causes a marked decrease in survival of PC12D cells. In addition, electrophoretic analysis of DNA demonstrates that H_2O_2 triggers DNA fragmentation in PC12D cells, indicating that there is an apoptotic component in H_2O_2-induced cell injury as DNA fragmentation has been regarded as an indication of apoptosis (Kaufmann *et al*., 2000). This cytotoxic effect of H_2O_2 is consistent with those reported by others. Satoh *et al*. (1996) reported that H_2O_2 significantly decreases the number of viable PC12 cells after 24 h. Besides, H_2O_2 causes membrane blebbing, nuclear condensation and DNA fragmentation, suggesting that the death PC12 cells via apoptosis (Satoh *et al*., 1996). The present result demonstrates that PC12D is also a useful model system to investigate apoptotic cell death induced by oxidative stressed.

When PC12D cells were preincubated with NGF, H_2O_2-induced cell death was significantly attenuated, suggesting that NGF protects PC12D cells against oxidative insult by H_2O_2. NGF-induced neuroprotection against free radical-insult in both dorsal root ganglion and PC12 cells has been well documented (Jackson *et al*., 1990; Goins *et al*., 1999; Guo & Mattson, 2000). The neuroprotective effect of NGF is based on the hypothesis that NGF induce the free radical-detoxifying mechanisms such as catalase activity, as catalase has been shown to be able to converse hydrogen peroxide to

molecular oxygen and water in vitro (Jackson *et al*., 1990).

Pretreatment of PC12D cells with MC14 significantly enhanced the NGF-induced protection. According to the hypothesis of the neuroprotective effect of NGF described above, MC14 may promote survival against H_2O_2-insult by amplifying the NGF-induced free radical detoxifying mechanisms. Since MC14 alone also exhibits protective effect against the cytotoxic injury induced by H_2O_2, it may be able to directly activate the catalase, or other detoxifying mechanisms. Besides, the possibility of MC14 to directly scavenge the free radicals, like the action of some antioxidants such as vitamin C (Path, 1990), should not be ruled out.

Using the H_2O_2-induced PC12D cell model, the present study demonstrates that MC14 can effectively increase the number of viable cells under the oxidative stress, and protect cells against H_2O_2-induced apoptosis. In addition, MC14 also significantly promotes the neuroprotective effect of NGF against H_2O_2 insult. This finding and the neuronal survival supporting effect described in section 6.3 strongly suggest that MC14 is a promising candidate as a therapeutic agent to treat Alzheimer’s disease and other neurodegenerative diseases.

Chapter VII

Neurite-Regenerating Activity of MC14

In adult animals, most of neurons are postmitotic. The loss of neurons due to injury or diseases such as Alzheimer's disease (AD) cannot be replaced. However, nerve cells can regenerate severed neuritic processes to re-establish synaptic connection (Nicholls *et al*., 1992). NGF is one of the most important neurotrophic factors responsible for stimulating the regeneration of neurites from the injured neurons so as to reinnervate the damaging synaptic connections. Synapses are sites where activity in neuronal circuits are modulated. Synaptic signaling is believed to play important roles in regulating neuronal survival and structural plasticity during development (Oppenheim, 1991; Lewin & Barde, 1996), and aberrant synaptic signaling is implicated in some pathological neurodegenerative conditions (Mattson *et al*., 1998). It has been suggested that the neurodegenerating process is likely to begin at the loss of synaptic connectivity in various disorders including AD (Terry *et al*., 1991; Dekosky *et al*., 1996). Accordingly, the regeneration of neuritic processes is a pivotal initial step for a functional recovery following neurodegeneration.

In chapter VI, the activity of MC14 to support and promote NGF-induced neuronal survival has been demonstrated. Further investigation of the activity of MC14 to regenerate neurite from the degenerating neuronal cells is important for understanding its applicability and effectiveness on recovering the degenerating nervous system caused by neurodegenerative diseases or injuries.

The objective of this study is to develop a neurite-degenerating model system by

culturing the neuronal PC12D cells in NGF-deficient condition, and use this experimental paradigm to study whether the treatment of MC14 under NGF-deficient condition would alleviate neurite degeneration, and promote neurite regeneration. In addition, the mechanically neurite-sheared neuronal PC12D cell model was used to further examine the neurite-regeneration promoting effect of MC14 on neurite-severed neurons. The study would provide a comprehensive understanding and important insight on the therapeutic application of MC14 for the treatment of neurodegeneration caused by diseases or injuries in the nervous system.

7-1. Materials and Methods

7-1-1. Cell culture and preparation of neuronal PC12D cells

PC12D cells were maintained at 37° C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated horse serum (HS), 5% fetal bovine serum (FBS), 100 U/ml penicillin, 200 μg/ml streptomycin and 25 μg/ml ampicillin under a water-saturated atmosphere of 5% CO_2. The neuronal PC12D cells were prepared by seeding the naïve PC12D cells on collagen-coated 24-well culture plate with a cell density of $1x10^5$ per well. After 24 h incubation, the medium was changed to fresh medium containing 50 ng/ml NGF and further incubated for 48 h to obtain the neuronal PC12D cells.

7-1-2. Neurite degeneration of neuronal PC12D cells, and the effect of MC14 on attenuating neurite degeneration under NGF-deficient condition

After the neuronal PC12D cells were rinsed for 2 times with fresh DMEM, the medium was replaced with DMEM supplemented with 1% HS in NGF-deficient

concentrations ranging from 0.4-10 ng/ml, and the cells were further incubated in 72 h. In addition, 50 ng/ml NGF and NGF-untreated cultures were used as positive and untreated controls, respectively. Neurite degeneration of the neuronal PC12D cells was determined by monitoring the proportion of intact neurite-bearing cells after 24, 48 and 72 h exposure to NGF-deficient condition using a phase-contrast microscope with 200X magnification. Processes with length longer than 2 diameters of cell body were counted as neurites while the cells bearing the degenerated or disintegrated neurites shorter than two diameters of their cell bodies were not counted as neurite-bearing cells. For each datum point, the mean value was calculated from 4 random-field observations of 2 independent experiments and a minimum of 100 cells per field were counted.

To determine the attenuating effect of MC14 under NGF-deficient condition, neuronal PC12D cells were treated with 1.5 μg/ml MC14 at the time of exposing to deficient NGF concentrations (0.4-10 ng/ml) in DMEM supplemented with 1% HS. To determine the effect of MC14-pretreatment against neurodegeneration under NGF-deficient condition, naïve PC12D cells were treated with or without 6.25 μg/ml MC14 in the presence of 50 ng/ml NGF for 6 days (with medium renewal twice for every 72 h) to prepare MC14-pretreated and MC14-unpretreated neuronal PC12D cells, respectively. These two groups of cells were than washed with fresh medium, and then incubated in the deficient concentrations of NGF in the absence of MC14 for further 4 days.

7-1-3. Neurite-regeneration promoting activity of MC14

Two model systems were used to investigate the neurite-regeneration promoting effect of MC14. For the first model system, neurite-degenerating PC12D cells were induced by NGF-deficient treatment. Briefly, neuronal PC12D cells were rinsed with fresh DMEM for 2 times and then incubated in 0.4 ng/ml NGF in DMEM supplemented

with 1% HS for 48 h. Thereafter, cells were treated with a second-dose of MC14 (0.2-0.8 μg/ml) and NGF (2 ng/ml) for 48 h. To determine the effect of NGF, another group of cultures were treated with a second-dose of NGF (2-50 ng/ml) only. Cells with regenerated neurites longer than two diameters of their cell bodies were counted as neurite-regenerated cells.

Regarding the second approach for examining the neurite-regenerating activity of MC14, the mechanically neurite-sheared cells were used. Neurites of the neuronal PC12D cells were prepared as described above, neurites of the neuronal PC12D cells were sheared from the cell bodies by trituation as described by Twiss & Shooter (1995). In brief, medium was aspirated, and the cell processes were sheared by trituation with medium using a 5-ml glass pipette. Cells were centrifuged at 1000 rpm, and washed for 3 times with fresh medium by repeated centrifugation-resuspension. The neurite-sheared cells were then plated at $5x10^3$ cells per well on a 96-well culture plate in complete medium containing 0-50 ng/ml NGF in the presence or absence of 3 μg/ml MC14. After 48 h incubation, the proportion of cells with regenerated neurites was determined. Cells with regenerated neuritic processess 2-time longer than their cell bodies were counted as neurite-regenerated cells.

7-1-4. Observation of cells and phase-contrast photography

The neurite-regenerated cells were observed using an Olympus phase-contrast microscope with 200-400X magnification. Photo-slides were taken using an Olympus SC-35 type camera. Images were scanned and transformed into computer file using Polaroid Polascan 35/LE system. Contrast of the image was adjusted if necessary.

7-1-5. Statistical analysis

Each datum point is the mean ± SD of four random-field observations from two independent experiments. Significant difference was determined by the Student's *t*-test. $P<0.05$ was considered to have significant difference.

7-2. Results

7-2-1. Neurite degeneration in NGF-deficient medium

Neuronal PC12D cells have been demonstrated to be able to survival in the presence of 50 ng/ml NGF in serum-free medium (Chapter VI). In this study, an experimental paradigm was used to induce neurite degeneration without significant cell death by treating the neuronal PC12D cells in deficient NGF concentration in low serum-supplemented (1% HS) medium. As shown in Fig. 7-1, significant reduction of long neurite-bearing cells was observed after the neuronal PC12D cells were exposed to NGF-deficient medium from 0.4-10 ng/ml for 24 h compared with positive control. Approximately 40% reduction of neurite-bearing cells was recorded after the cells were exposed to 10 ng/ml NGF for 72 h. Under microscopic observation, neuronal PC12D cells extended long neurites in 50 ng/ml NGF (Fig. 7-2a,b). After treated with NGF-deficient medium (0.4-10 ng/ml) for 72h, however, the disrupted and degenerated

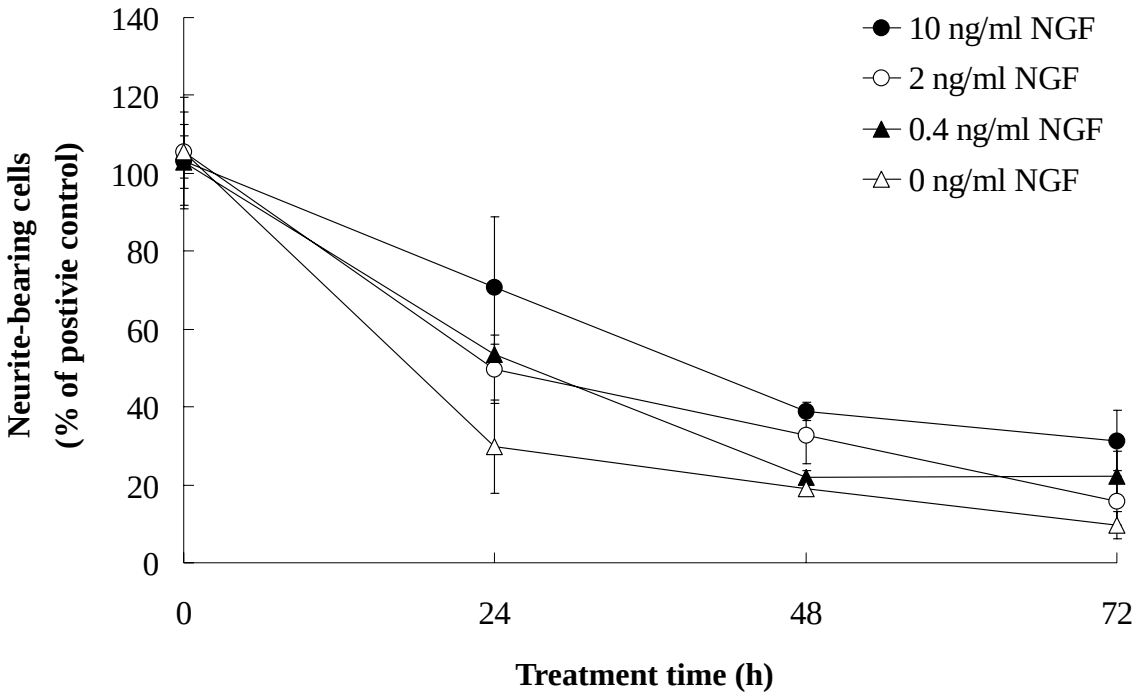


Fig. 7-1. Neurite degeneration of neuronal PC12D cells in NGF-deficient medium supplemented with 1% horse serum. Neuronal PC12D cells were incubated in the indicated concentrations of NGF for 72 h. Neurite-bearing cells were counted at 0, 24, 48 and 72. Neurite-bearing cells are expressed as a percentage relative to positive control (50 ng/ml, 100%). Data are given as mean (n=4) ± SD from 2 independent experiments.

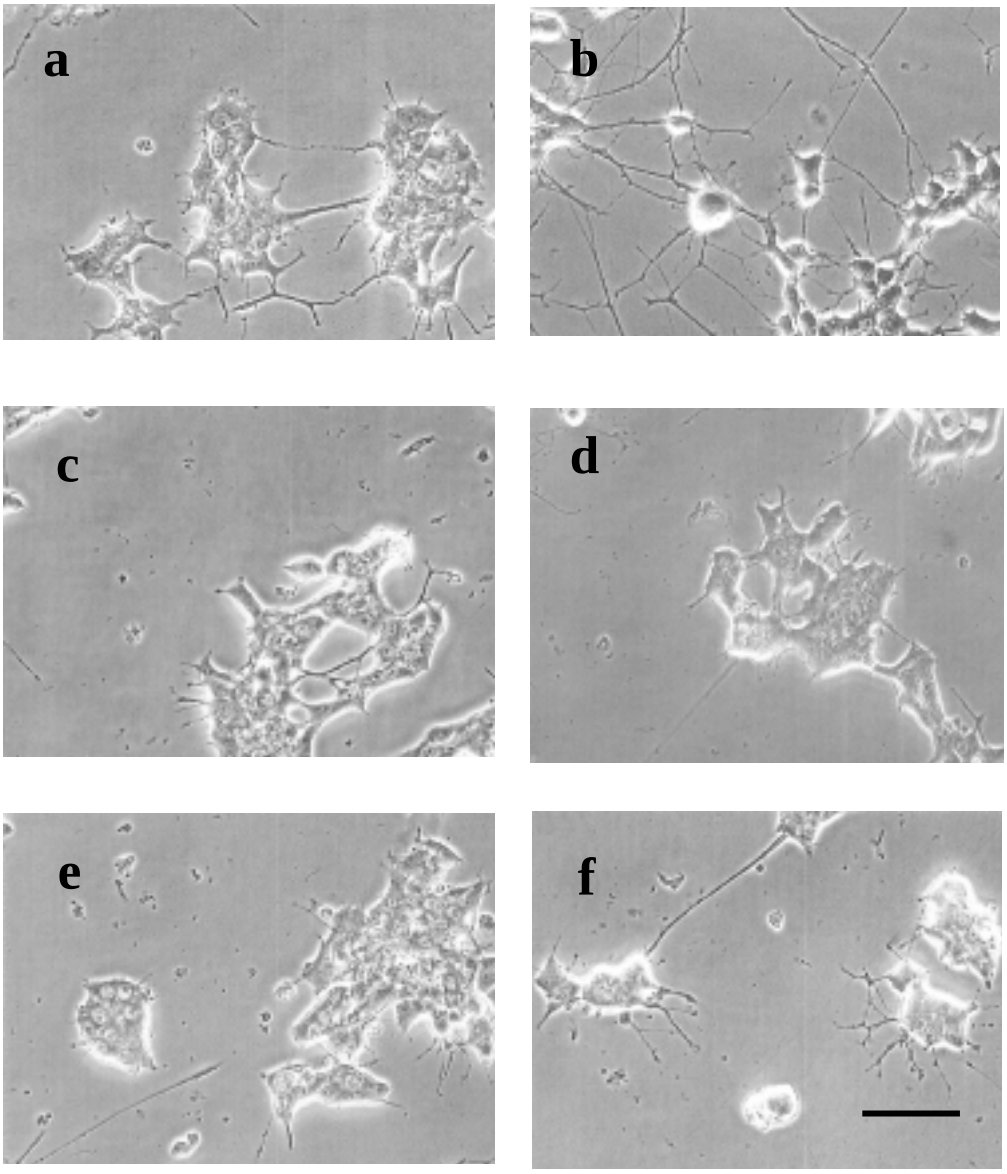


Fig. 7-2. Neurite degeneration of neuronal PC12D cells incubated in NGF-deficient medium. PC12D cells were differentiated into neuronal PC12D cells after treated with 50 ng/ml for 48 h (a), and then exposed to 50 ng/ml NGF (b), 10 ng/ml NGF (c), 2 ng/ml NGF (d), 0.4 ng/ml NGF (e) or 0 ng/ml NGF (f) for 72 h. Scale bar: 50 μm.

neurites were observed (Fig. 7-2c-f). It should be noted that many neurite fragments were observed in the medium, indicated that the observed reduction of neurite-bearing cells was attributed by the neurite degeneration, rather than neurite retraction.

7-2-2. Effect of MC14 on attenuating neurite-degeneration induced by NGF-deficient treatment

After PC12D cells were neuronally differentiated, they were subjected to NGF-deficient medium (0.4-10 ng/ml) supplemented with or without MC14. The proportion of neurite-bearing cells was monitored at 0, 24, 48 and 72 h. In the medium supplemented with 1.5 μg/ml MC14 at 10 ng/ml NGF, the decreasing rate of the neurite-bearing cell number was significantly lowered compared with that of MC14-untreated control (Fig. 7-3a). Comparing the cells treated with 50 ng/ml (positive control), there was only 25% reduction of neurite-bearing cells for the cells treated with MC14 after 72 h, whereas more than 70% reduction was recorded for those cells treated with NGF only. Significant differences of the neurit-bearing cell number in the MC14-treated and untreated cultures were also recorded in 2 and 0.4 ng/ml NGF (Fig. 7-3b,c). These results indicated that the presence of MC14 could attenuate neurite degeneration under NGF-deficient condition. The phase-contrast photographs clearly show that greater number of MC14-treated cells maintained their long neurites in NGF-deficient conditions compared with the corresponding MC14-untreated controls (Fig. 7-4).

7-2-3. Protective effect of MC14 pretreatment of cells from neurite-degeneration under NGF-deficient condition

To corroborate the neurite-protective effect of MC14 before the induction of neurite

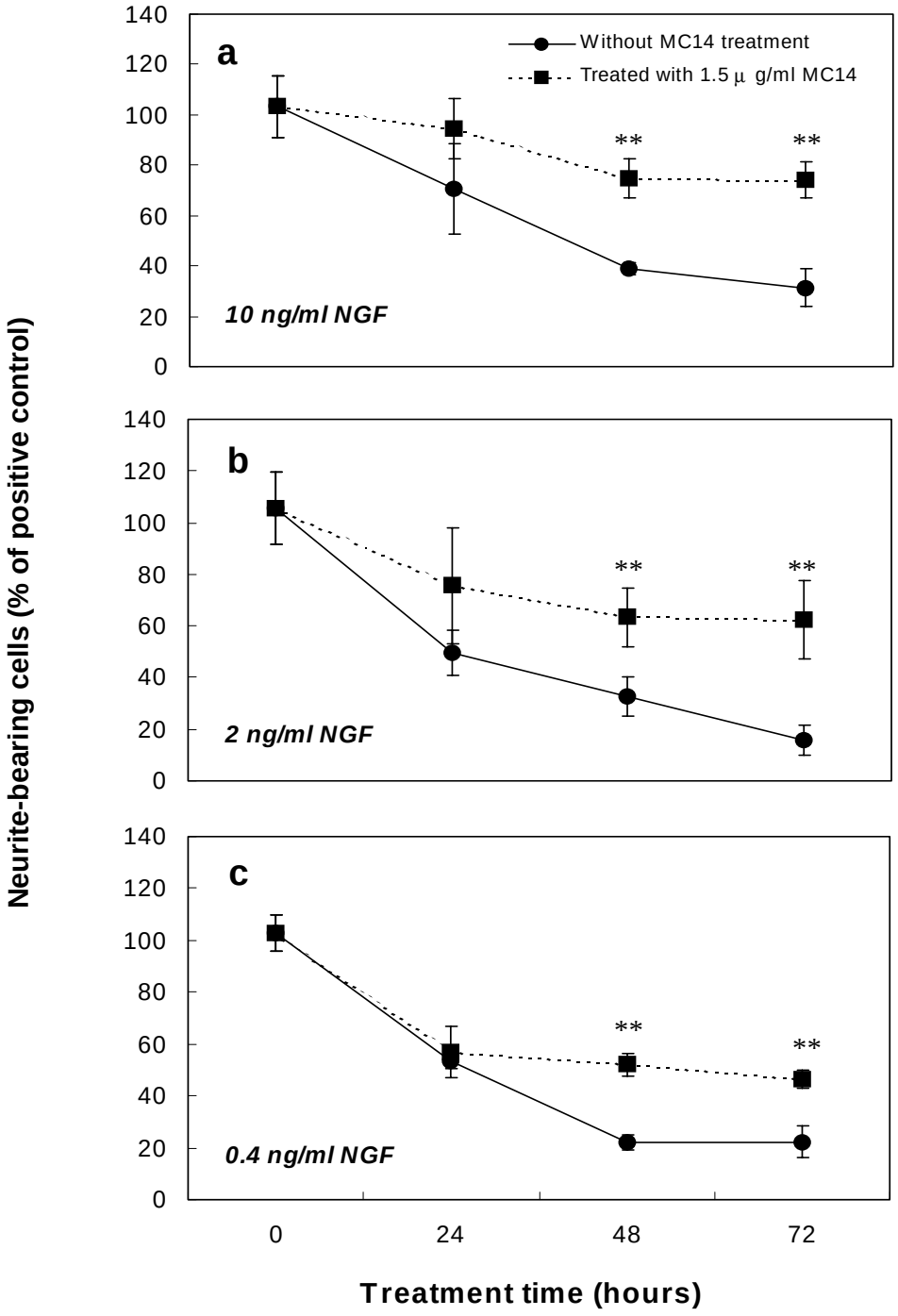


Fig. 7-3. Effect of MC14 on attenuating neurite degeneration of neuronal PC12D cells under NGF-deficient condition. Neuronal PC12D cells were treated with the indicated concentrations of NGF with 1.5 μg/ml MC14 for the indicated time. Neurite-bearing cells are expressed as a percentage relative to positive control (50 ng/ml NGF, 100%). Data are given as mean (n=4) ± SD from 2 independent experiments. Significant difference from MC14-untreated control: **$P < 0.01$ (Student's *t*-test).

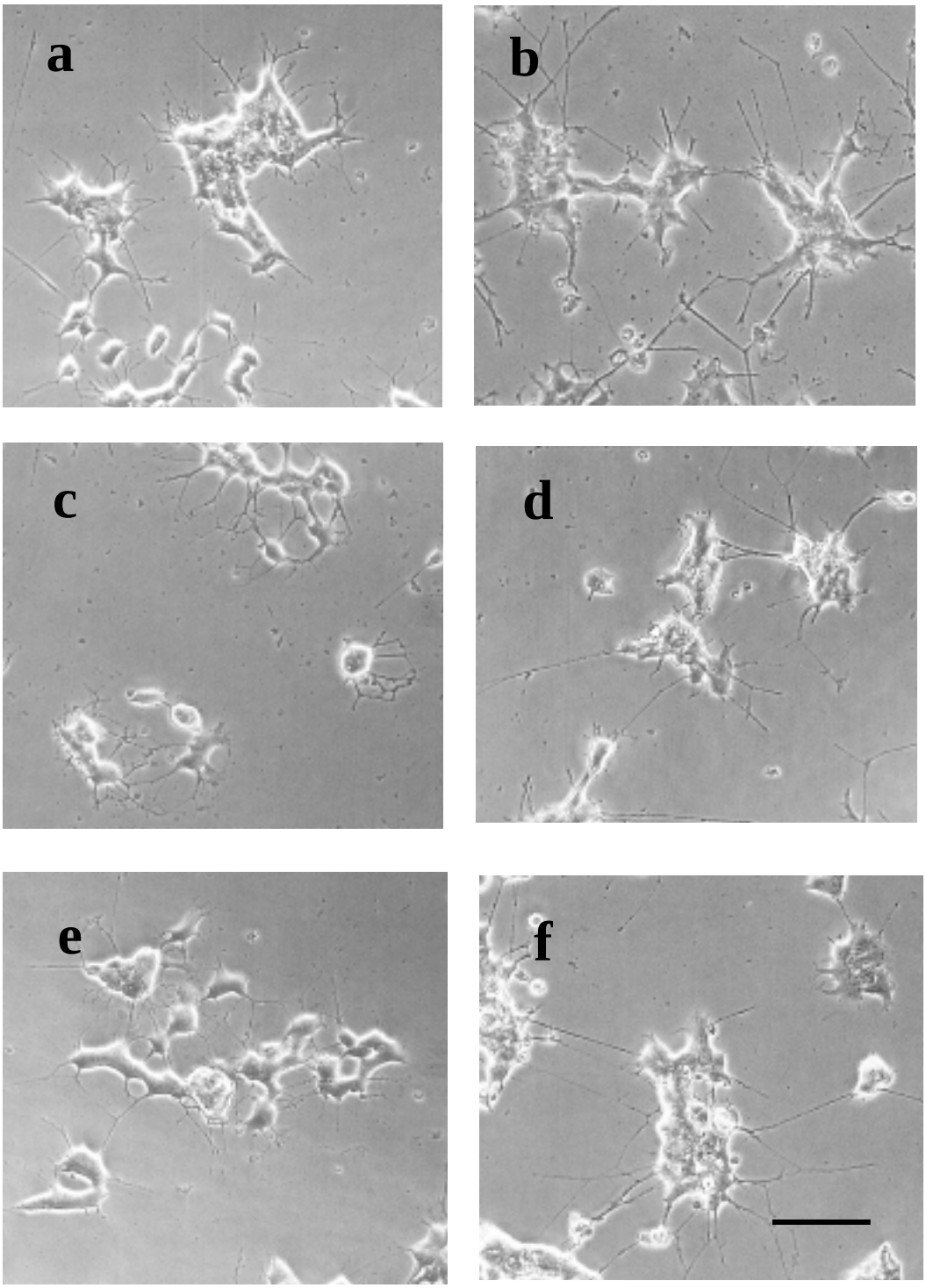


Fig. 7-4. Effect of MC14 on attenuating neurite degeneration under NGF-deficient condition. Neuronal PC12D cells were incubated in the absence (a, c, e) or presence (b,d,f) of 1.5 μg/ml MC14 at 10 ng/ml (a, b), 2 ng/ml (c, d) or 0.4 ng/ml NGF (e, f) for 72 h. Scale bar: 50 μm.

degeneration, naïve PC12D cells were treated with MC14 during their differentiation period before subjecting to NGF deficiency. After MC14-pretreatment, the cell medium was changed to fresh medium containing the deficient concentration of NGF only, then the cells were further incubated for 4 days. Comparing with the control group without MC14 pretreatment, the extent of neurite-degeneration was significantly reduced for the MC14-preteated cells in the deficient concentration of 0.4-10 ng/ml NGF (Fig. 7-5), suggesting that MC14 pretreatment of naïve PC12D cells appeared to increase the resistance of cells against neurite degeneration under NGF-deficient condition.

7-2-4. Neurite regenerating effect of MC14

Two modeling systems were used to examine the neurite regenerating capability of MC14 on neurite-degenerated cells. For the first modeling system, neurite-degenerated cells were induced by incubating the neuronal PC12D cells in 0.4 ng/ml NGF for 48 h. Subsequently, a second dose of NGF (2, 10, 50 ng/ml), or MC14 (0.2, 0.4, 0.8 μg/ml) plus 2 ng/ml NGF was added to the neurite-degenerated cultures. After 48 h, neurite-bearing cells were counted again. As shown in Fig. 7-6, a dose-dependent enhancement of neurite-bearing cells was recorded in the cultures treated with MC14 compared with those treated with 2 ng/ml NGF only. It should be noted that the combined effect of 0.8 μg/ml MC14 and 2 ng/ml NGF was higher than that of the second dose of 50 ng/ml NGF. The morphological changes of cells before and after the second dose treatments are shown in Fig. 7-7. After the neuronal PC12D cells were treated with 0.4 ng/ml NGF, most of the neurites were degenerated, leaving a few neurite-bearing cells (Fig. 7-7a). After the second-dose treatment with 2 ng/ml NGF, no significant neurite regeneration was observed (7-8b). However, the number of neurite-bearing cells was markedly

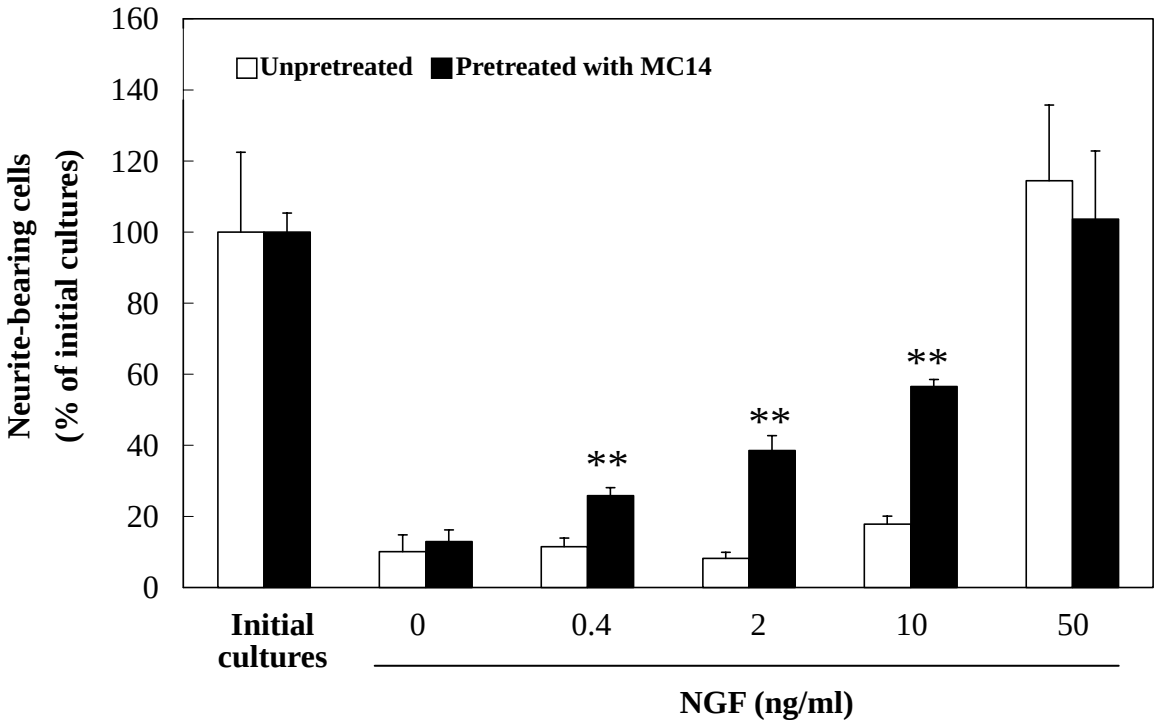


Fig. 7-5. Neuroprotection of MC14-pretreatment of PC12D cells against neurite-degeneration. Naïve PC12D cells were treated with (MC14-pretreated PC12D group) or without (MC14-unpretreated group) 6.25 μg/ml MC14 in the presence of 50 ng/ml NGF for 6 days (initial cultures). Media of both groups of cells were changed with fresh media containing the indicated concentrations of NGF only. Proportion of neurite-bearing cells were counts after 4 days of incubation. Neurite-bearing cells are expressed as a percentage relative to positive control (50 ng/ml NGF, 100%). Data are given as mean ± SD (n=4). Significant difference between MC14-pretreated and unpretreated cells are analysed by Student's *t*-test with ** $p < 0.01$.

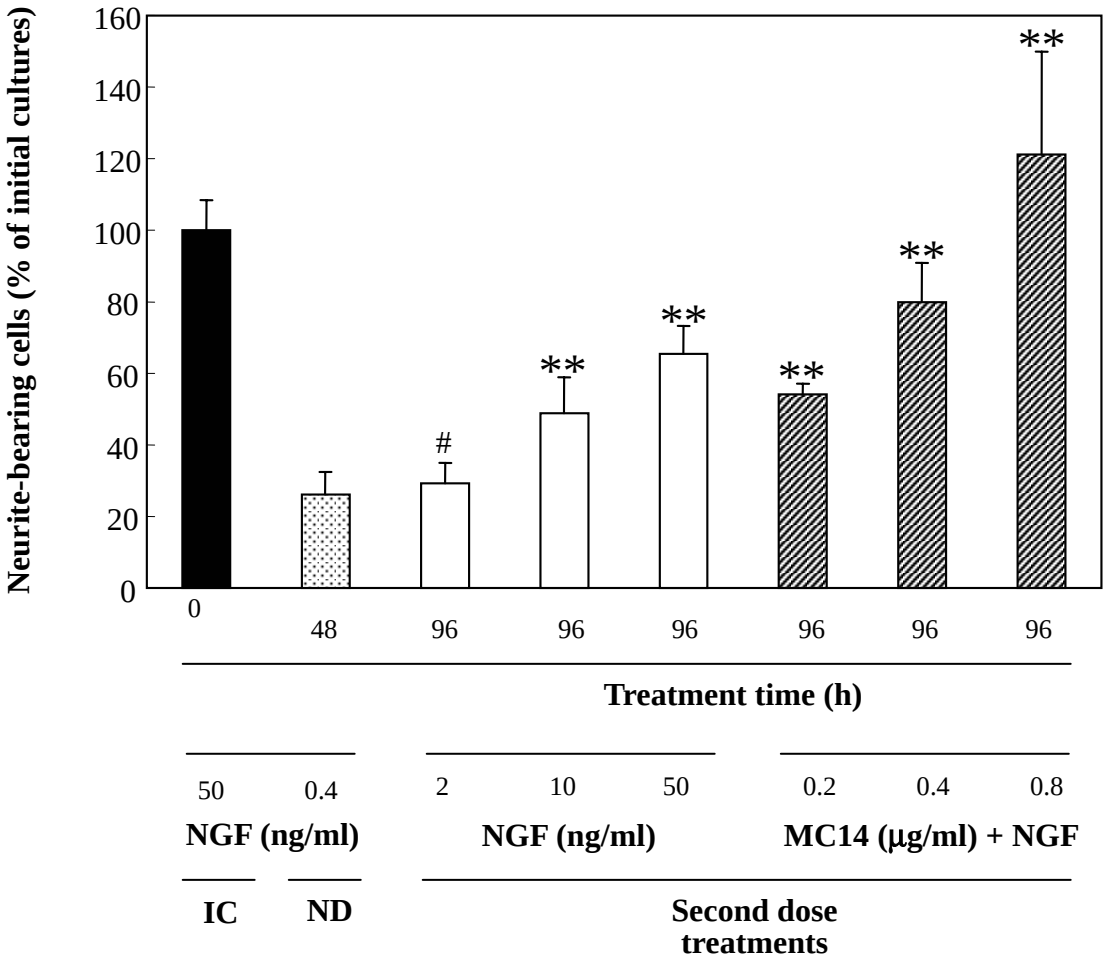


Fig. 7-6. Neurite-regeneration activity of MC14 and NGF. Neurite degenerated cells (ND) were induced by exposing the neuronal PC12D cells (initial cultures, IC) in 0.4 ng/ml NGF for 48 h. Cells were then treated with the second dose of the indicated concentrations of NGF, or MC14 plus 2 ng/ml NGF for further 48 h. Neurite-bearing cells are expressed as a percentage relative to that of initial cultures before NGF-deficiency. Data are given as mean (n=4) ± SD. Significant difference from MC14-untreated control (#): ** $p < 0.01$ (Student's *t*- test).

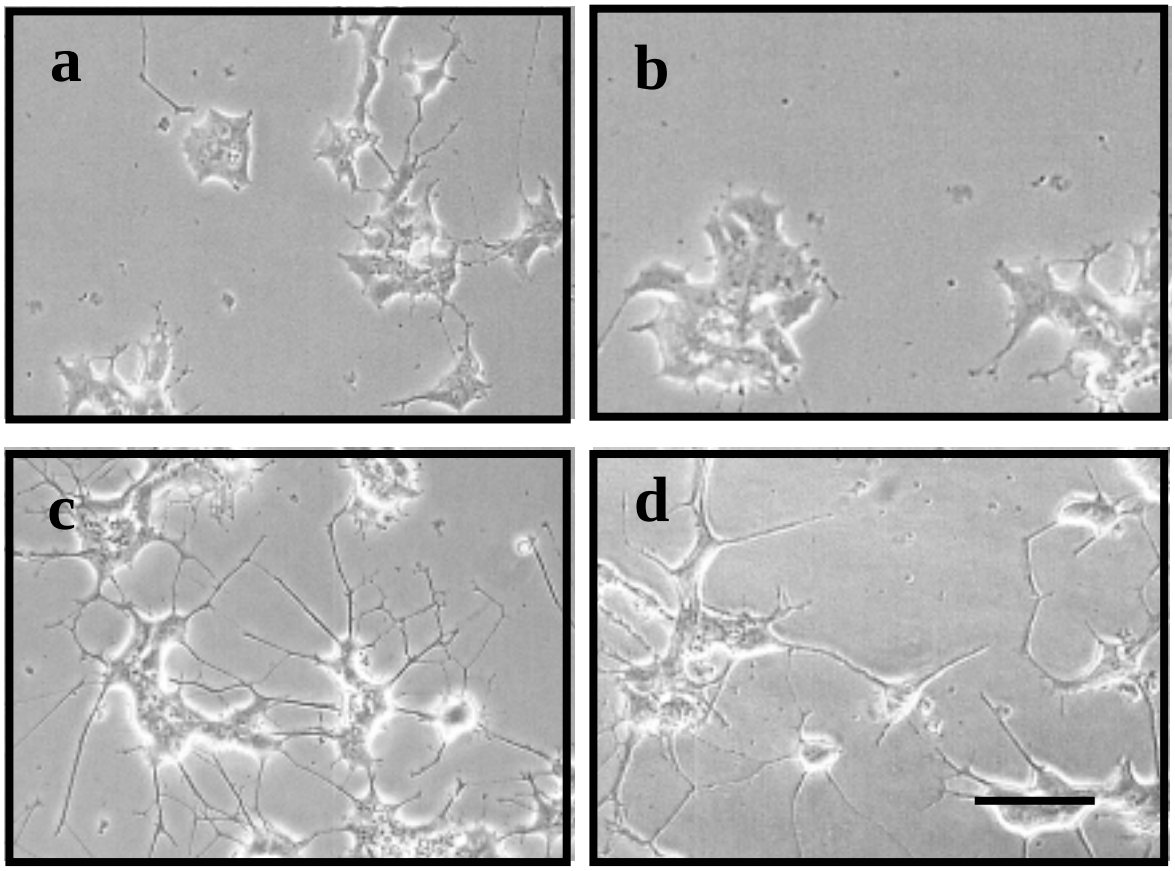


Fig. 7-7. Neurite-regenerating effect of MC14 and NGF. Neurite-degenerated cells were exposed to 0.4 ng/ml NGF for 24 h (a), and then treated with a second dose of 2 ng/ml NGF (b), 0.8 μg/ml MC14 plus 2 ng/ml NGF (c) or 50 ng/ml NGF (d) for further 48 h. Scale bar: 50 μm.

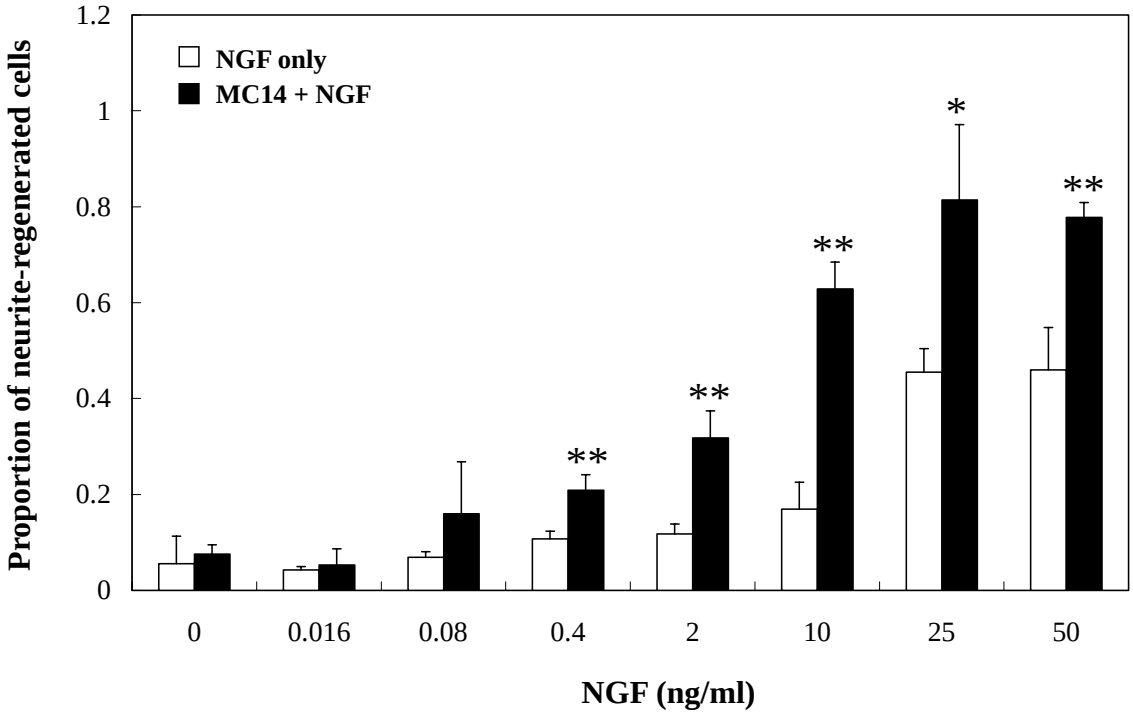


Fig. 7-8. Neurite-regeneration promoting effect of MC14 on neurite-sheared PC12D cells. Neurites of the neuronal PC12D cells were sheared by trituation and the resulting neurite-sheared cells were treated with or without 3 μg/ml MC14 in the presence of the indicated concentrations of NGF for 48 h. Data are given as mean (n=4) ± SD. Significant difference from the NGF-only control: *$P < 0.05$ and **$P < 0.01$ (Student's *t*-test).

increased after the cells were treated with the second doses of 2 ng/ml NGF and 0.8 μg/ml MC14 (Fig 7-7c), while the number of neurite-bearing cells was higher than that of the second dose of 50 ng/ml NGF (Fig. 7-7d).

To get a further insight into the neurite regenerating effect of MC14 on neurite-severed neurons, the neuronal PC12D cells were sheared and subsequently treated with or without MC14 in the presence of NGF ranging from 0.016-50 ng/ml for 48 h. Measurement of neurite-regenerated cells showed that the treatment of 3 μg/ml MC14 in the presence of 0.4-50 ng/ml NGF resulted in significant enhancements of neurite-regenerated cells compared with the respective MC14-untreated control (Fig. 7-8). Besides, the MC14-enhanced neurite regeneration showed a dose-depended manner of NGF. The maximum enhancement of neurite regeneration by 4-fold was recorded after the neurite-sheared cells were treated with 3 μg/ml MC14 in the presence of 10 ng/ml NGF. The morphologies of neurite-sheared cells and neurite-regenerated cells after various treatments of MC14 and NGF are shown in Fig. 7-9. It should be noted that MC14 stimulated the cells to extend more branching and longer neurites compared with the corresponding NGF-only controls.

Taken together, these data clearly demonstrate that MC14 can effectively promote neurite regeneration from the mechanically sheared cells as well as the neurite degenerating cells induced by NGF deficient treatment.

7-3. Discussion

Substantial studies have shown that NGF-responsive neurons such as sympathetic neurons, cholinergic neurons and PC12 cells undergo neurite degeneration, followed by

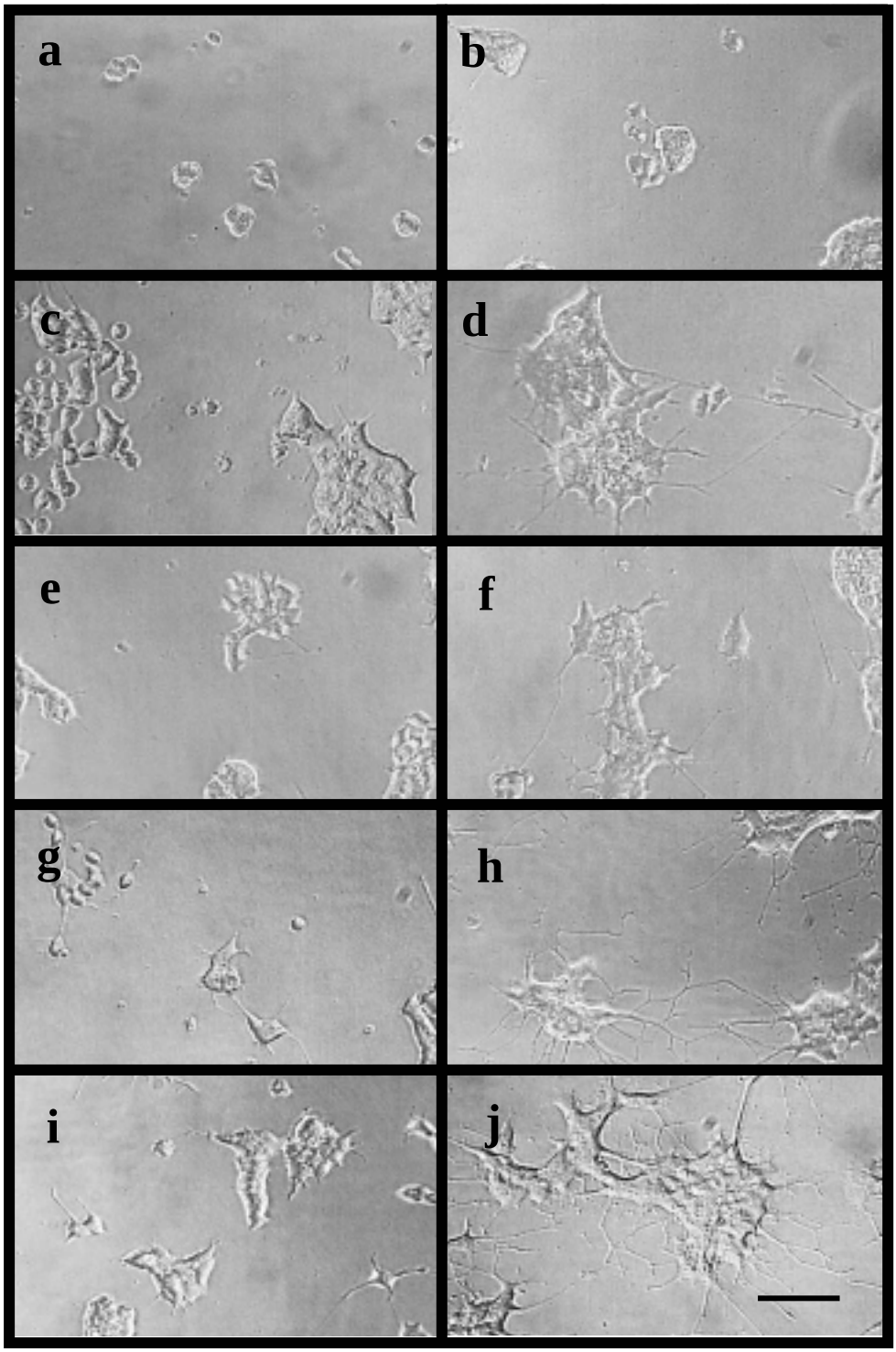


Fig. 7-9. Neurite regeneration promoting activity of MC14 on neurite-sheared PC12D cells. Morphology of the neuronal PC12D cells after sheared by trituation (a). The neurite-sheared cells treated with neither MC14 nor NGF (b), 0.4 ng/ml NGF only (c), 3 μg/ml MC14 and 0.4 ng/ml NGF (d), 2 ng/ml NGF only (e), 3 μg/ml MC14 and 2 ng/ml NGF (f), 10 ng/ml NGF only (g), 3 μg/ml MC14 and 10 ng/ml NGF (h), 25 ng/ml NGF only (i), 3 μg/ml MC14 and 25 ng/ml NGF (j) for 48 h. Scale bar: 50 μm.

cell death after NGF deprivation (Batistatou & Greene, 1991; Mesner *et al*., 1995; Araki & Wurtman, 1998; McGinty *et al*., 2000). It has also been reported that the removal of NGF from the medium of neuronally differentiated PC12 cells leads to a sequential events, including reduction in metabolic activity, increase in the expression of amyloid precursor protein, neurite degeneration, DNA fragmentation and finally apoptotic cell death (Batistatou & Greene, 1991; Araki & Wurtman, 1998). It has been described in chapter VI that complete removal of NGF in serum-free medium will cause neuronal PC12D cell death within 24 h. In the present experimental paradigm, neuronal PC12D cells were incubated in NGF-deficient medium supplemented with 1 % horse serum. Under these conditions, the neuronal PC12D cells do not completely degenerate or commit apoptotic cell death, but exhibited neurite disruption within the experimental period of 72 h. This phenomenon resembled that of neuronal cells cultured in NGF-deficient medium as reported by others (Batistatou & Greene, 1991; Araki & Wurtman, 1998). Thus, this model system was used to study the neurite-regenerating activity of MC14.

Under this experimental paradigm, substantial proportion of cells exhibited neurite disruption after exposed to the NGF-deficient concentration (0.4-10 ng/ml) from 24-72 h. Although the molecular mechanisms involved in the observed neurite degeneration of the neuronal PC12D cells by NGF-deficient treatment is remained to be clarified, a recent report has described that NGF deprivation would be followed by the formation of the persistent actin-depolymerizing factor and cofilin-actin rods that span the diameter of neurites, disrupt the microtubules and cause degeneration of the distal neurite without killing the cultured hippocampal neuron (Minamide *et al*., 2000). The neurite degeneration in the neuronal PC12D cells induced by NGF-deficient treatment might

result from the similar mechanisms as the biochemical events of neuronal PC12D cells are resemble those of neuronal cells (Greene & Tischler, 1976; Katoh-Semba *et al.*, 1987).

Treatment of neuronal PC12D cells with MC14 in NGF deficient medium significantly reduces the rate of neurite degeneration, implying that MC14 may effectively attenuate neurite degeneration caused by the deficiency of neurotrophic support in the nervous system. Apart from alleviating neurite degeneration, the pretreatment of naïve PC12D with MC14 during their differentiation period appears to increase the resistance of cells against neurite degeneration under NGF-deficient condition as significant lower rate of neurite degeneration was observed in the MC14-pretreated cells after the subsequent incubation in NGF-deficient medium. These results imply that the pretreatment of MC14 may have protective effect against neurite degeneration, and that MC14 might have neuroprotective effect if treated before the onset of neurodegenerative disease. Although the signaling pathway involved in the observed protective effect of MC14 on PC12D cells remains to be elucidated, it is speculated that the pretreatment of naïve PC12D cells with MC14 during their differentiation period might stabilize the microfilaments in the neurites so as to increase their resistance against degeneration in NGF deficient medium.

It has been demonstrated by others that the treatment of neurite-degenerated neuronal cells with NGF or certain growth factors can induce their neurite regeneration (Green *et al*., 1983; Davies *et al*., 1997; Schicho *et al*., 1999). Consistently, neuronal PC12D cells response to the second dose of NGF to regenerate neurite. More strikingly, the combined effects of MC14 and a relative low concentration of NGF (2 ng/ml) markedly enhance the neurite regeneration compared with the effect of NGF alone.

On the other hand, it has been demonstrated that the resupplement of NGF to neurite-severed cells can rescue neuronal sympathetic neurons and PC12 cells from apoptosis or neurite degeneration (Cai *et al*., 1999). To investigate whether MC14 could promote neurite regeneration after neuronal injury, the mechanically neurite-sheared neuronal PC12D cells, used as a neurite-severed neuronal model, were treated with MC14 in the presence of various NGF concentrations. The result clearly demonstrates that MC14 can significantly promote NGF-induced neurite regeneration from the neurite-severed cells. Additionally, this finding provides a supporting evidence for the activity of MC14 to effectively enhance neurite-regeneration from injured neurons.

Collectively, the neurite-regeneration promoting activity of MC14 suggests that it might enhance axonal regeneration, which is an important initial step for the subsequent reinnervation with the neurons after neuronal injury. On the molecular basis, a previous report showed that NGF stimulates the synthesis of structural neural proteins such as growth-associated protein and Tα1 α-tubulin in neuron cell body (Mohiuddin *et al*., 1995). Recent studies have also demonstrated that the enhancement of vesicle fusion and insertion of newly synthesized membrane at distal neurite portion and at the growth cone allow expansion of neurite elongation (Phenninger & Friedman, 1993; Horner & Gage, 2000). During the regeneration of neurite from the severed-neuron, NGF plays a crucial role in guiding the neurite to reinnervate and reconnect the synapse (Horner & Gage, 2000). Based on these findings, it is plausible that MC14 might facilitate the regeneration process by enhancing the rate of microtubules assembling and reconstruction, leading to the observed neurite regeneration from PC12D cells. Alternatively, MC14 might mediate the signaling pathway of NGF which regulates the level of intracellular messengers such as cyclic AMP to enhance the phosphorylation

state of cytoskeleton-associated proteins to regenerate the neurites from the severed cells.

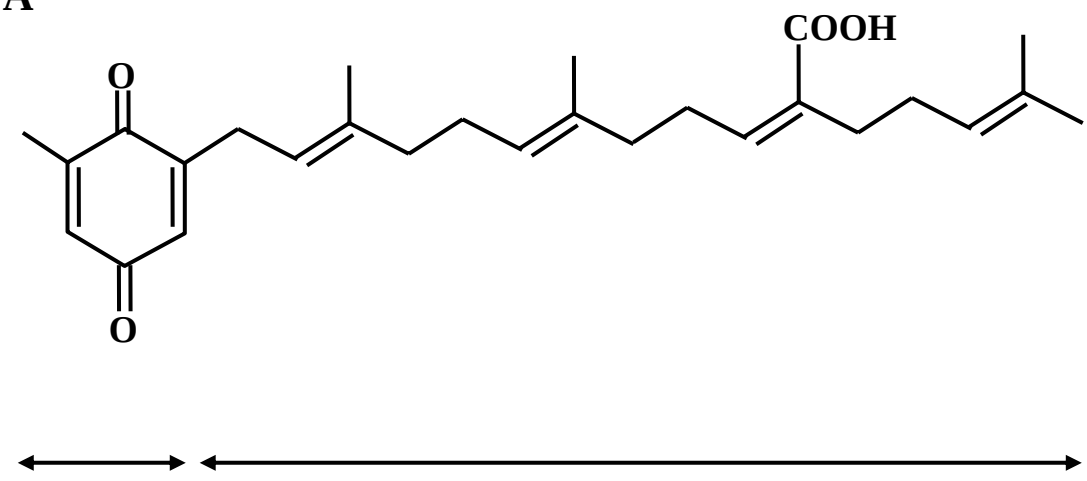
An *in vivo* study has suggested that the insufficient supply of NGF may lead to neurite degeneration and disruption of synaptic connection of cholinergic neurons to hippocampus, resulted in retardation of memory and cognitive functions (Fischer *et al*., 1987). It has also been demonstrated that NGF infusion to aged monkey basal forebrain has can effectively reinnervate the neurons to hippocampus, which plays a key role in memory and cognitive function (Kordower *et al*., 1994). Therefore, MC14 might be a promising agent to promote NGF-induced repair of neuronal network to improve memory and cognitive functions for neurodegenerative diseases or injury. Besides, the present study provides a more direct evidence to show that MC14 may attenuate neurodegeneration. Furthermore, MC14 exhibits neuroprotective effect, implying that it may help to delay the onset or slow down the progression of neurodegenerative diseases such as AD.

Chapter VIII

Structure-Activity Relationship of MC14 and Its Analogues

The development of therapeutic drugs for the treatment of neurodegenerative disorders such as Alzheimer's disease (AD) has become an urgent issue, due to the lack of effective therapeutic drugs and to the expected rise in the number of AD patients in the near future. In chapter II, the structural elucidation by NMR spectroscopic analysis of MC14 has been described in detail, and the chemical structure of MC14 was elucidated (Fig. 2-6). MC14 is composed of a carotenoid-like moiety and a quinone moiety (Fig. 8-1). Although MC14 has a promising NGF-potentiating activity, its relatively complex structure renders its chemical synthesis not commercially feasible. The present study aimed to identify the part of the MC14's structure that is essential to the molecule's NGF-potentiating effect. To accomplish this, the activity of 1,4-benzoquinone (the fundamental quinone) and eight carotenoid analogues were examined. In addition, the structure-activity relationship of quinones was investigated in order to study the effect of their structural modification on NGF-mediated neurite outgrowth-enhancing activity. The results may provide information important to the development of a comparatively simple synthetic chemical as a therapeutic agent with high efficacy in treating neurodegenerative disease. Furthermore, the results may provide valuable information for delineating the mechanisms by which these biologically active substances act on NGF-mediated neurite outgrowth from PC12D cells.

A



Quinone moiety

Carotenoid-like moiety

B

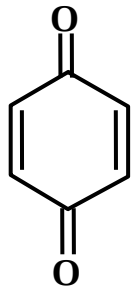


Fig. 8-1. (A) Chemical structure of MC14. MC14 is composed of a quinone moiety and a carotenoid-like moiety. (B) Chemical structure of 1,4-benzoquinone.

8-1. Materials and Methods

8-1-1. Materials

All quinone compounds were purchased from Adrich Chemical Co., while β-carotene and other carotenoids were kindly given by Dr. Tanaka, Faculty of Fisheries, Kagoshima University, Japan. All reagents and drugs were of analytical grade.

8-1-2. Cell culture and bioassay of neurite outgrowth-promoting activity

PC12D were maintained as described in chapter IV. When bioassay was conducted, the cells were seeded on a 96-well culture plate with a cell density of 5×10^3 cells per well. After 24 h incubation, the medium was changed to fresh medium containing appropriate test concentrations of various samples and 10 ng/ml NGF. In addition, the cells treated with 50 ng/ml NGF alone and 10 ng/ml NGF alone were designated as positive control and negative control, respectively. After 48 h incubation, the neurite outgrowth from PC12D cells was monitored using a phase-contrast microscope with 200X magnification. Processes with lengths longer than 2 diameters of the respective cell body were counted as neurites. For each datum point, the mean value was calculated from four random field observations, and a minimum of 100 cells per field were counted. The neurite outgrowth promoting activity was calculated as the proportion of neurite-bearing cells to the total number of cells in the same field.

8-1-3. Statistical analysis

Each datum point is the mean ± S.D. of four random field observations from two

replicate experiments. Significant differences from the negative control (10 ng/ml NGF) were determined by the Student's *t*-test. $P < 0.05$ was considered to be a significant difference.

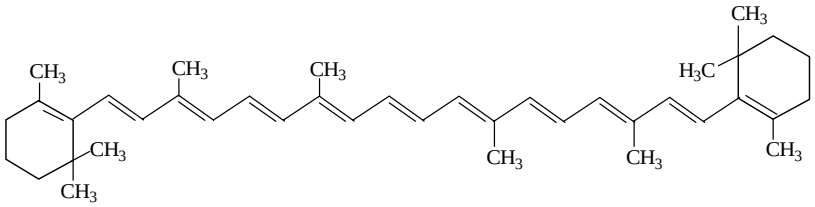
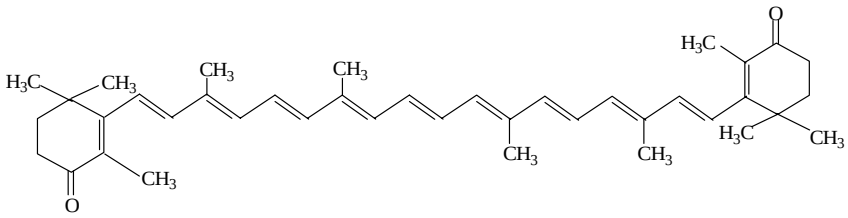
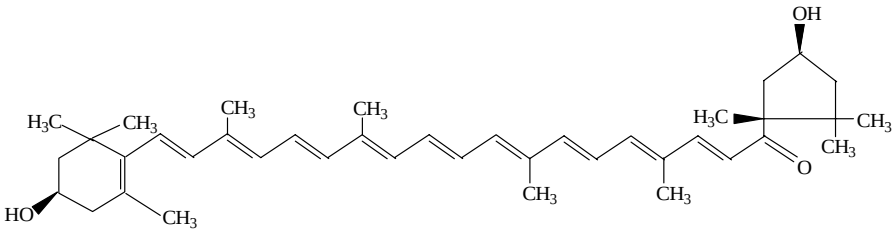
8-2. Results

As described in chapter II, the chemical structure of MC14 was elucidated to be sargaquinoic acid, which consisted of a quinone structure and a carotenoid side chain. To delineate the structurally essential moiety of MC14 molecule for its NGF-potentiating activity, 1,4-benzoquinone and carotenoids were used to represent the quinone and carotenoid moieties of the MC14 molecule, and their neurite outgrowth promoting activity were determined.

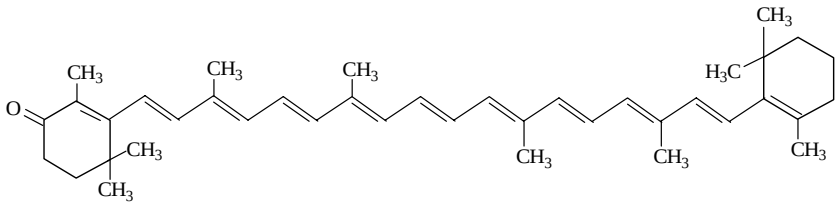
8-2-1. Neurite outgrowth promoting activity of carotenoids and 1,4-benzoquinone

Each carotenoid consisted of an alkyl-chain with at least 20 carbons to mimic the alkyl-chain of the MC14 molecule (Table 8-1). The bioassay results showed that none of the carotenoid compounds enhanced the proportion of neurite-bearing cells compared to the negative control, suggesting that the carotenoid moiety alone did not stimulate NGF-mediated neurite outgrowth from PC12D cells (Fig. 8-2). In contrast, 1,4-benzoquinone showed a significant enhancing effect on neurite outgrowth from PC12D cells. The proportion of neurite-bearing cells increased from 7% to 23% in the presence of 6.25

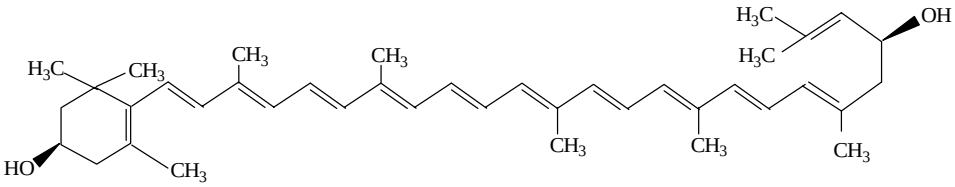
Table 8-1. Chemical structures of carotenoids tested in this study.

Carotenoids	**Chemical strcuture**
β-carotene	
Canthaxanthin	
Capsanthin	

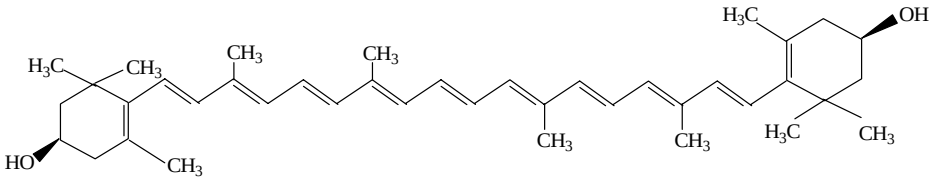
Echinenone



Rubixanthin



Zeaxanthin



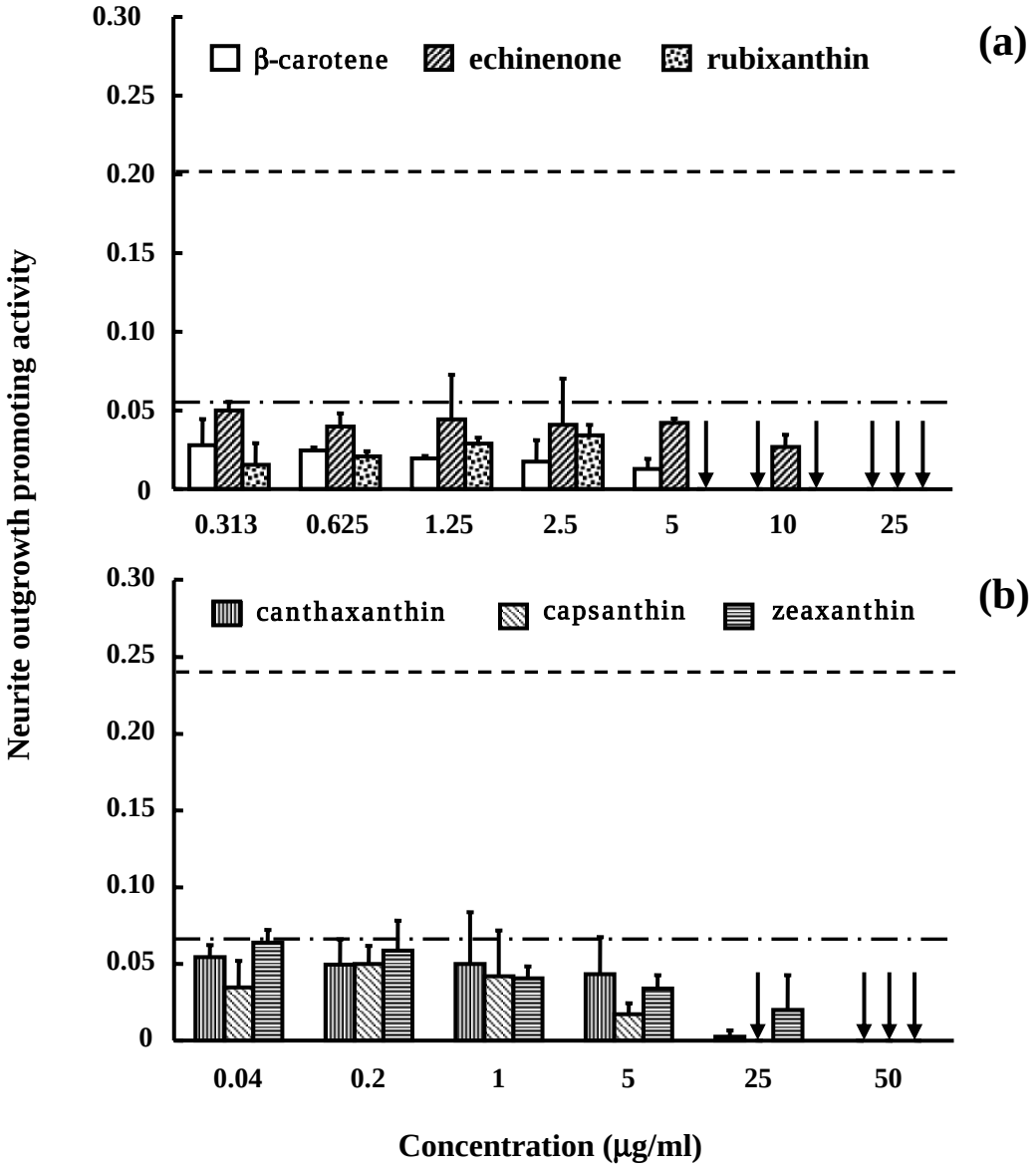


Fig. 8-2. Neurite outgrowth-promoting activity of (a) beta-carotene, echinenone, rubixanthin and (b) canthaxanthin, capsanthin, zeaxanthin. PC12D cells were treated with the indicated concentrations of tested chemicals in the presence of 10 ng/ml NGF for 48 h. Cells treated with 50 ng/ml NGF (- - - - - -) and 10 ng/ml NGF (— - — -) were used as positive control and negative control, respectively. Arrows indicate cytotoxicity.

μg/ml 1,4-benzoquinone, which is equivalent to a 260 % increase compared to the effect induced by the negative control (Fig. 8-3). The maximum effective concentration of 1,4-benzoquinone was the same as that of MC14, indicating similar levels of potency in enhancing neurite outgrowth from PC12D cells. Taken together, these results clearly demonstrated that the quinone moiety of the MC14 molecule might be the essential structure for NGF-potentiating activity.

The morphological appearance of cells activated by 1,4-benzoquinone is shown in Fig. 8-4e. Both the neurite length and the cell shape were similar to those of the cells stimulated by MC14. In respect of a cytotoxic effect on PC12D cells, both MC14 and 1,4-benzoquinone exhibited similar levels of cytotoxicity at the lowest toxic concentration of 12.5 μg/ml, at which the cells underwent apoptosis-like cell shrinkage. Comparing the maximum neurite outgrowth-enhancing activity, we found that 1,4-benzoquinone enhanced neurite outgrowth by 260% of the negative control while MC14 enhanced that by 500% of the negative control, indicating that 1,4-benzoquinone was less effective than MC14 in terms of NGF-potentiating activity. This may be due to the structural difference between the quinone moiety of MC14 and 1,4-benzoquinone.

8-2-2. Neurite outgrowth promoting activity of naturally occurring quinone compounds

In order to investigate the effects of substitute groups of 1,4-benzoquinone on neurite outgrowth-enhancing activity, 12 naturally occurring quinones with various substitute groups were selected, and bioassays were performed under the same conditions. The chemical structures of the quinones tested in the study were shown in Table 8-2. Among them, alizarin, lapachol and lawsone exhibited significant neurite outgrowth-enhancing

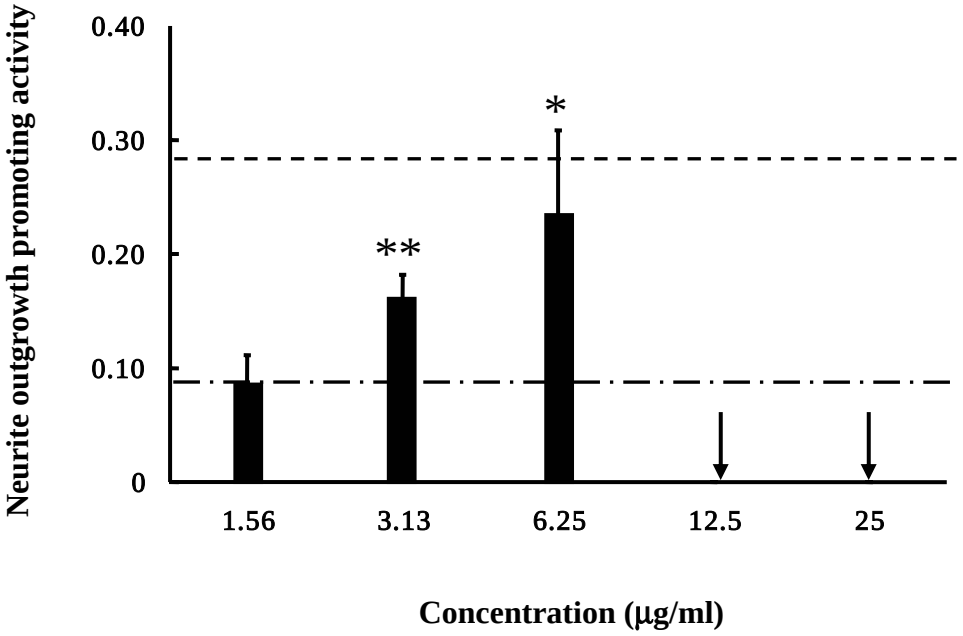


Fig. 8-3. Neurite outgrowth-promoting activity of 1,4-benzoquinone. PC12D cells were treated with the indicated concentrations of 1,4-benzoquinone in the presence of 10 ng/ml NGF for 48 h. Cells treated with 50 ng/ml NGF (- - - - - -) and 10 ng/ml NGF (— - — -) were used as positive control and negative control, respectively. Arrows indicate cytotoxicity. Significant difference from the negative control: *P < 0.05, *P* < 0.01 (Student's *t*-test).

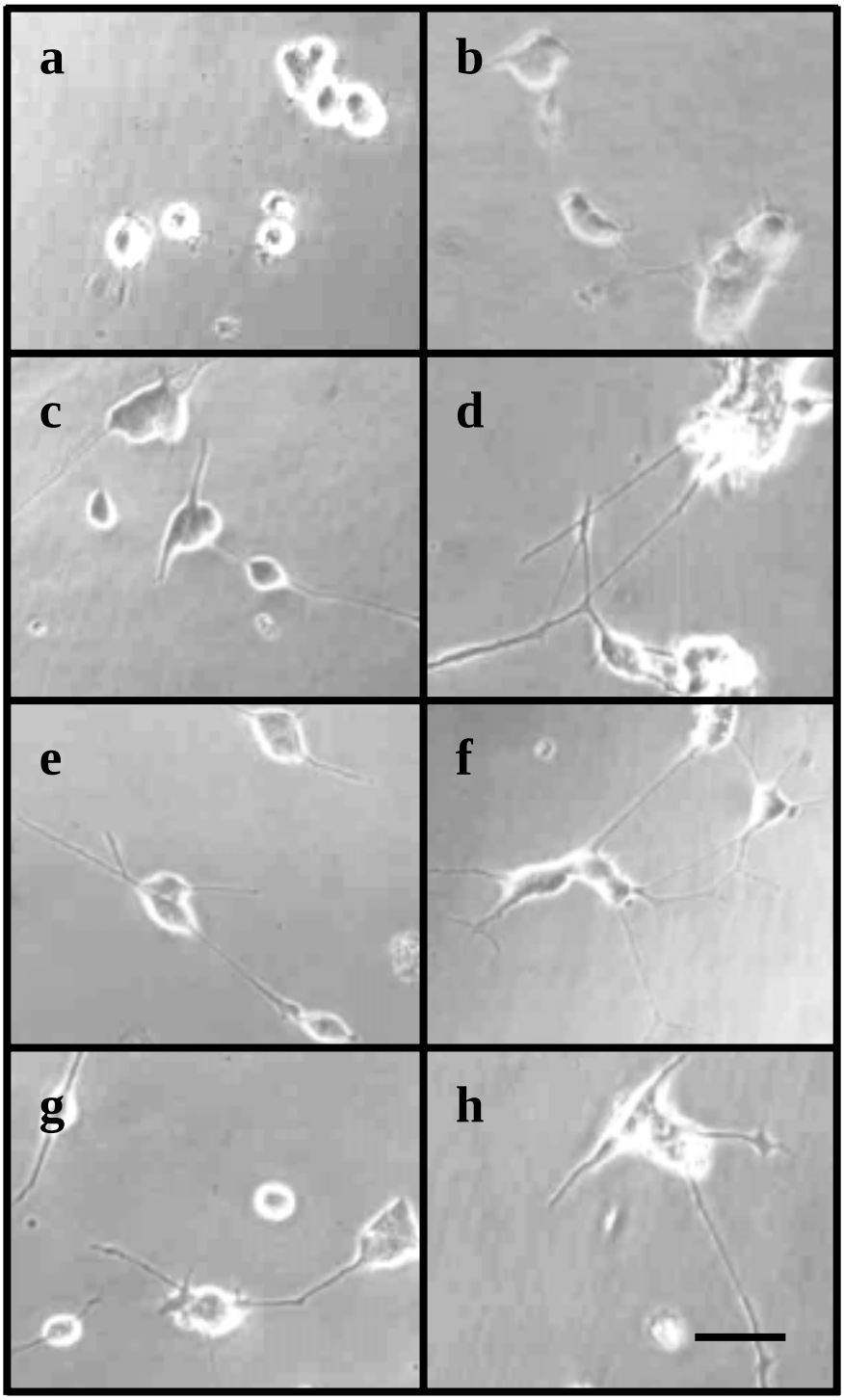
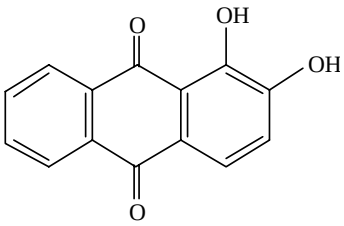
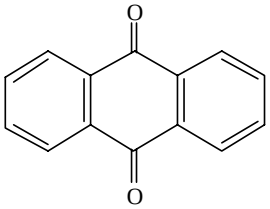
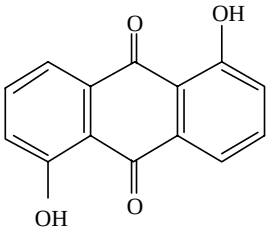
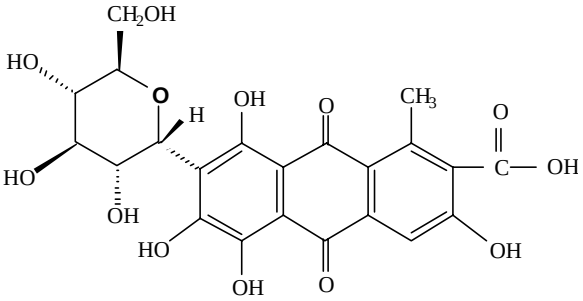
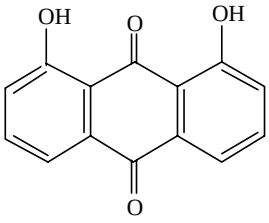
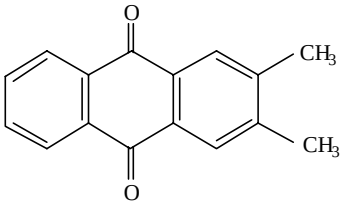
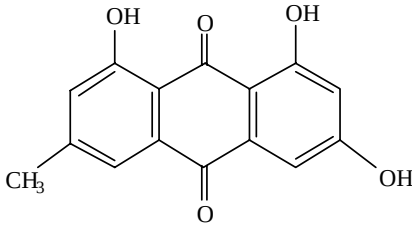
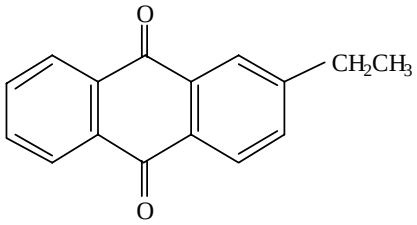
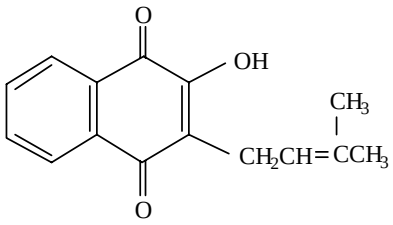
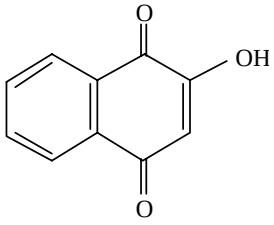
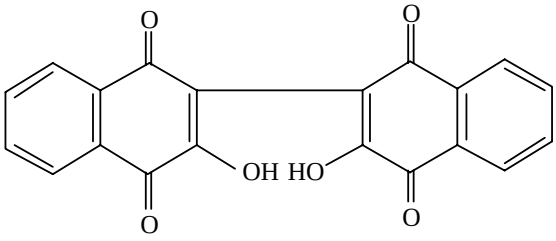
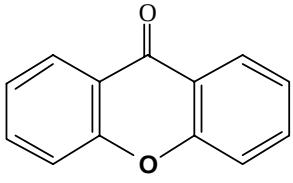


Fig. 8-4. Phase-contrast photomicrographs of PC12D after various treatments for 48 h. (a) MC14-untreated control culture (0 ng mL^{-1} NGF). (b) PC12D cells treated with 10 ng mL^{-1} NGF (negative control). (c) PC12D cells treated with 50 ng mL^{-1} NGF (positive control). PC12D cells treated with (d) 6.25 µg mL^{-1} MC14, (e) 6.25 µg mL^{-1} 1,4-benzoquinone, (f) 100 µg mL^{-1} lawsone, (g) 12.5 µg mL^{-1} alizarin or (h) 100 µg mL^{-1} lapachol in the medium containing 10 ng mL^{-1} NGF for 48 h. Bar = 25 µm.

Table 8-2. Chemical structures of quinones tested in this study.

Quinone compounds	Chemical formula	Chemical structure
Alizarin	$C_{14}H_8O_4$	
Anthraquinone	$C_{14}H_8O_2$	
Anthrarufin	$C_{14}H_8O_4$	
Carminic Acid	$C_{22}H_{20}O_{13}$	
1,8-dihydroxy-anthraquinone	$C_{14}H_8O_4$	
2,3-Dimethyl-anthraquinone	$C_{16}H_{12}O_2$	

Emodin	$C_{15}H_{10}O_5$	
2-Ethylanthra-quinone	$C_{16}H_{12}O_2$	
Lapachol	$C_{15}H_{14}O_3$	
Lawsone	$C_{10}H_6O_3$	
Lawsone dimer	$C_{20}H_{10}O_6$	
Xanthone	$C_{13}H_8O_2$	

effects on PC12D cells (Table 8-3). The maximum activities of lapachol, alizarin and lawsone were 265%, 325% and 329%, respectively, of the negative control. The highest effective concentrations for alizarin, lapachol and lawsone were 12.5 μg/ml, 50 μg/ml and 100 μg/ml, respectively, indicating that alizarin is the most potent of these three neurite outgrowth enhancers. The photomicrographs of PC12D cells treated with these active quinones are shown in Fig. 8-4f-h. In an attempt to investigate the neurotrophic action of the quinones alone, an identical concentration range of the quinones was examined in the absence of NGF. It was revealed that none of the quinones induced neurite outgrowth from PC12D cells (data not shown), implying that these quinones, like MC14, could significantly enhance the NGF-mediated neurite outgrowth from PC12D cells but do not mimic the effect of NGF.

8-3. Discussion

Naturally occurring quinones consist of a large variety of compounds, including numerous pigments, vitamin K and electron carriers such as plastiquinone and ubiquinone (Smith *et al*., 1997). These compounds are known to have antihemorrhagic effect on blood clotting, antitumour activity and electron transport function within the membranes of chloroplasts and mitochondria (Stryer, 1996). This study demonstrates that three naturally occurring quinones can enhance NGF-mediated neurite outgrowth. In the literature, there are few reports on the chemicals capable of potentiating NGF action. Pamela *et al*. (1995) reported that a synthetic compound, AIT-082, which is a purine derivative, exhibited neurite outgrowth-enhancing activity on PC12h cells. A fungal

Table 8-3. **Neurite outgrowth-promoting activity of various quinone compounds on PC12D cells. PC12D cells were treated with the indicated concentrations of quinones in the presence of 10 ng mL^{-1} NGF for 48 h. Neurite outgrowth-promoting activity was calculated as described in Materials and methods. Cells treated with 50 ng mL^{-1} and 10 ng mL^{-1} were served as positive control (PC) and negative control (NC), respectively. Standard deviation for each mean value (n = 4) is indicated in parenthesis. CT = cytotoxicity.**

Quinone compounds	Neurite outgrowth-promoting activity								
	NGF concentration (ng mL^{-1})								
	50	10							
	Quinone concentration (μg mL^{-1})								
	0 (PC)	0 (NC)	3.13	6.25	12.5	25	50	100	200
Alizarin	0.278 (0.029)	0.077 (0.015)	0.090 (0.024)	**0.164* (0.033)**	**0.250** (0.015)**	CT	CT	CT	CT
1,4-anthraquinone	0.278 (0.029)	0.077 (0.015)	0.039 (0.010)	0.037 (0.009)	0.059 (0.028)	0.046 (0.021)	0.121 (0.046)	CT	CT
Anthrarufin	0.278 (0.029)	0.077 (0.015)	0.059 (0.006)	0.061 (0.017)	0.046 (0.015)	0.058 (0.009)	0.043 (0.016)	0.038 (0.008)	CT
Carminic acid	0.278 (0.029)	0.077 (0.015)	0.056 (0.023)	0.070 (0.022)	0.098 (0.045)	0.064 (0.015)	0.097 (0.016)	CT	CT
1,8-dihydroxyanthraquinone	0.278 (0.029)	0.077 (0.015)	0.062 (0.003)	0.084 (0.013)	0.053 (0.015)	0.069 (0.056)	0.103 (0.026)	0.122 (0.017)	CT
2,3-dimethylanthraquinone	0.278 (0.029)	0.077 (0.015)	0.097 (0.003)	0.117 (0.046)	0.082 (0.053)	0.110 (0.001)	0.082 (0.006)	0.114 (0.042)	CT
Emodin	0.278 (0.029)	0.077 (0.015)	0.051 (0.020)	0.049 (0.041)	CT	CT	CT	CT	CT
2-ethylanthraquinone	0.278 (0.029)	0.077 (0.015)	0.097 (0.032)	0.059 (0.023)	0.107 (0.053)	0.107 (0.024)	0.101 (0.050)	CT	CT
Lapachol	0.278 (0.029)	0.077 (0.015)	0.079 (0.029)	0.081 (0.025)	0.081 (0.014)	0.096 (0.047)	**0.204** (0.045)**	**0.133* (0.035)**	CT
Lawsone	0.278 (0.029)	0.077 (0.015)	0.127 (0.035)	**0.141* (0.038)**	**0.150** (0.024)**	**0.156** (0.028)**	**0.229* (0.083)**	**0.253** (0.036)**	CT
Lawsone dimer	0.278 (0.029)	0.077 (0.015)	0.064 (0.011)	0.045 (0.012)	0.047 (0.004)	0.115 (0.039)	CT	CT	CT
Xanthone	0.278 (0.029)	0.077 (0.015)	0.034 (0.007)	0.039 (0.008)	0.062 (0.023)	0.037 (0.018)	CT	CT	CT

Value significantly different from negative control is bold: * $p < 0.05$, ** $p < 0.01$

metabolite, NG-061, a derivative of phenylacetic acid hydrazide, and some unknown soluble factors from rat basophilic leukemia cells were also reported to have neurite outgrowth-stimulating effects on PC12 cells (Suzuki *et al*., 1998; Ito *et al*., 1999). In addition, a neuropeptide somatostatin and nardosinone from a Chinese medicinal plant were demonstrated to significantly enhance neurite outgrowth from PC12D cells (Ferriero *et al*., 1994; Li *et al*., 1999). Most of these natural and synthetic compounds are comparatively complex and not amenable to commercial exploitation. Previous chapters have demonstrated that MC14 is promising therapeutic agent for the treatment of neurodegenerative disorders by virtue of its strong NGF-potentiating activity. However, its chemical structure is also comparatively complex. The analysis of structural moiety of MC14 molecule responsible for the observed NGF-potentiating activity will help identifying a chemical with a relatively simple structure suitable for industrial chemical synthesis, and provide information for the structure modification of NGF-potentiating chemicals. The neurite outgrowth enhancing activity of carotenoids and 1,4-benzoquinone clearly demonstrated that only the quinone moiety of the MC14 molecule is structurally essential for NGF-potentiating activity.

The stronger activity of MC14 than that of 1,4-benzoquinone may be contributed to the presence of substitute groups of quinone moiety of the MC14 molecule. To test this hypothesis, the NGF-potentiating activity of 12 naturally occurring quinones with various substitute groups were examined. The results show that the substitutions of hydroxyl groups on the quinone molecule is likely to enhance the NGF-potentiating activity. By comparing the activities of alizarin and anthraquinone, the presence of 2 hydroxyl groups on alizarin seems to be essential for its activity. The arrangement of these 2 hydroxyl groups on anthraquinone molecule also significantly influences the activity as neither

anthrarufin nor 1,8-dihydroxyanthraquinone shows activity, indicating that only the substitutions of two hydroxyl groups at the 1’ and 2’-positions of anthraquinone structure exhibits the neurite outgrowth-promoting activity.

On the other hand, the presence of a hydroxyl group at the 1’-position of lawsone and lapachol is also expected to be crucial to their activity, since it is the unique structure for both compounds. In addition, the loss of activity of lawsone dimer may be attributed to the steric effect of its bulky structure, suggesting that molecular configuration is also one of the effects determining the neurite outgrowth-enhancing activity of quinone. Taken together, the positions of the hydroxyl groups on the quinone molecule may play a key role in NGF-potentiating activity, and they might be the binding sites of the quinone to NGF molecule or to NGF receptor.

In comparing the neurite outgrowth-enhancing effect of MC14 and the active quinones, it was speculated that MC14 might first interact and bind to the NGF molecule by its quinone moiety to form a NGF-MC14 complex. The alkyl-chain of MC14 then facilitates the attachment of this complex to the hydrophobic plasma membrane of PC12D cell. Alternatively, the MC14 might bind to NGF molecule and in turn enhance the binding of NGF to its TrkA receptor, which subsequently up-regulates the NGF-mediated signal cascades that promote transcription events, leading finally to neurite outgrowth. In addition, MC14 might prolong the interaction time of NGF and its receptor by the binding of the MC14 side-chain on the plasma membrane.

The present study may serve toward the development of a chemical with a simple structure and a potent NGF-enhancing effect for the pharmacological treatment of neurodegenerative diseases.

Chapter IX

Novel Effect of Vitamin K_1 (Phylloquinone) and Vitamin K_2 (Menaquinone) on Promoting Nerve Growth Factor-Mediated Neurite Outgrowth from PC12D Cells

During a comparative analysis of the structural analogues of MC14, it was found that vitamin K_1 and K_2 are structurally similar to MC14 (Fig. 9-1). Since the K vitamins have been showed to have age-dependent survival-promoting activity on cultured CNS neurons (Nakajima *et al*., 1993), study of the effect of K vitamins on NGF-potentiating activity will be important for further understanding their neuronal activities. In this study, the bioassays of the NGF-mediated neurite outgrowth promoting activity on PC12D cells were performed. In addition, several specific inhibitors were used to investigate the signaling pathways involved in the K vitamins-enhanced neurite outgrowth on PC12D cells.

9-1. Materials and Methods

9-1-1. Materials

Vitamin K_1 and K_2 were purchased from Sigma. All other materials used were described in chapter IV.

9-1-2. Cells culture and neurite outgrowth promoting assay

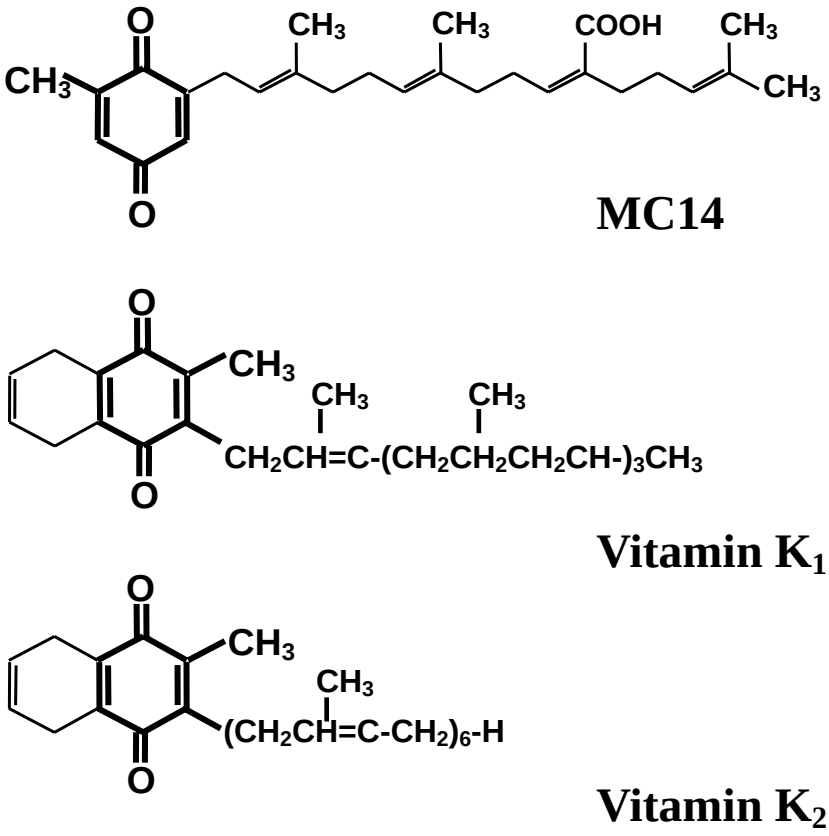


Fig. 9-1. Chemical structure of MC14, vitamin K_1 and vitamin K_2. The common moiety shared by these compounds is indicated by bold line.

PC12D cells were cultured as described in chapter IV. When bioassay was conducted, cells were harvested and plated at a density of 5 X 10^3 per well on a collagen-coated 96-well plate in complete medium. After 24 h, NGF (0-50 ng/ml) and 100 μg/ml vitamin K compounds (the highest concentration without observable cytotoxic effect on PC12D cells) were added to the cells. After 48 h, the proportion of neurite-bearing cells was determined using a phase-contrast microscope with 200X magnification. For each datum point, the mean value was calculated from six random field observations of two replicate experiments, and a minimum of 100 cells per field were counted.

9-1-3. Effect of various inhibitor on activity of K vitamins

For studying various inhibitors' effects, cells were preincubated for 1 h in the presence or absence of the inhibitors before the addition of NGF and vitamin K compounds. After incubation for 48 h, the proportion of neurite-bearing cells was determined.

9-2. Results

Treatment of PC12D cells with K vitamins in the presence of 2.5-50 ng/ml NGF significantly increased the proportion of neurite-bearing cells over those treated with NGF alone, and the concentration-response curve of NGF was shifted to the left by 100 μg/ml vitamin K_1 and K_2 (Fig. 9-2). In addition, the K vitamins significantly enhanced the proportion of cells with neurite at the optimally-effective concentration of NGF (50 ng/ml). However, vitamin K compounds alone could not induce neurite outgrowth from PC12D cells. On the other hand, the K vitamins enhanced neurite outgrowth from

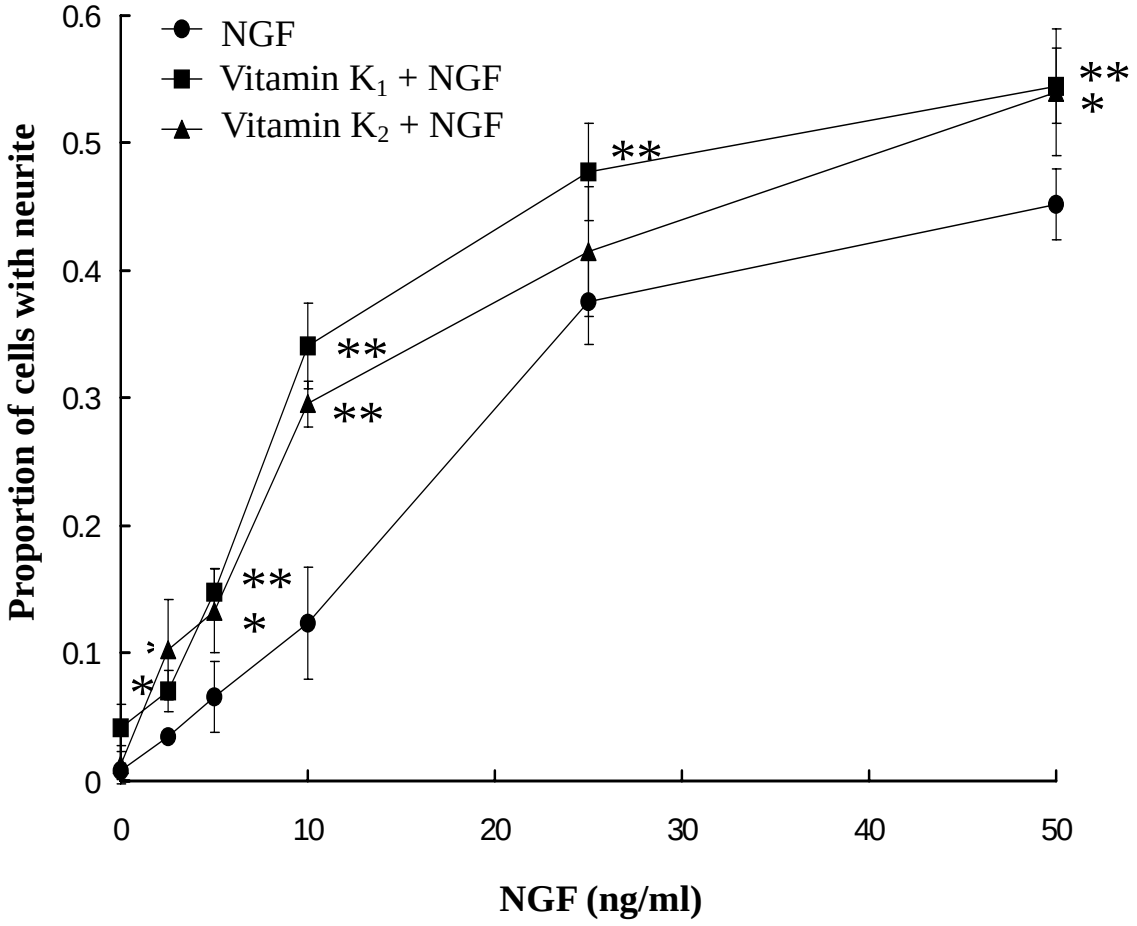


Fig. 9-2. Dose-response curve of the neurite outgrowth from PC12D cells for NGF, NGF plus 100 μg/ml vitamin K_1 and NGF plus 100 μg/ml vitamin K_2. Each point represents the mean ± SD (n=6) from two replicate experiments. Significant difference from the NGF-only control: *P < 0.05; **P < 0.01 (Student's t-test).

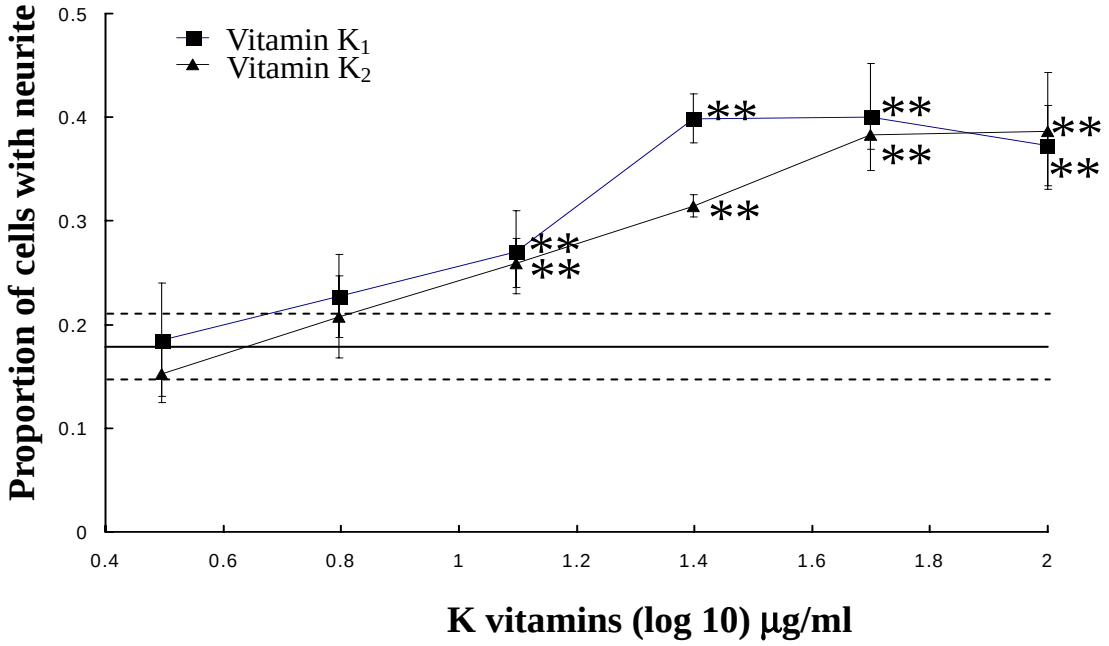


Fig. 9-3. Dose-response curve of the neurite outgrowth enhancing effect of vitamin K_1 and K_2 on PC12D cells in the presence of 10 ng/ml NGF. Each point represents the mean ± SD (n=6) from two replicate experiments. The horizontal solid line represents the mean value for the cultures treated with 10 ng/ml NGF alone; the dotted lines represent the SD (n=6) of this value. Significant difference from the NGF-only control: **P < 0.01 (Student's *t*-test).

PC12D cells in the presence of 10 ng/ml NGF in concentration-dependent manner (Fig. 9-3). To investigate the mechanisms of K vitamins, the effect of specific inhibitors for MAPK kinase, PKA and PKC on K vitamins-stimulated neurite outgrowth from PC12D cells was tested. As shown in Fig. 9-4, the PKA inhibitor at 5 nM substantially blocked the K vitamins-enhanced neurite outgrowth from PC12D cells. Pretreatment of cells with 10 μM PD98059, a specific MAPK kinase inhibitor, almost completely inhibited the enhancing effect of vitamin K compounds. In contrast, no significant inhibition of neurite outgrowth was observed for those cells treated with chelerythrine chloride.

9-3. Discussion

Naturally occurring vitamin K compounds consist of vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone). Although their physiological roles (e.g. blood coagulation, bone metabolisms and osteoporosis) have been well documented (Suttie, 1991), their roles in the central nervous system (CNS) remain to be clarified. The present study describes that vitamin K_1 and K_2 show significant NGF-mediated neurite outgrowth promoting activity. Concerning the study of their mechanisms of actions, the effect of K vitamins is substantially blocked by the pretreatment of cells with a specific PKA inhibitor, suggesting that vitamin K_1 and K_2 enhance NGF-induced neurite outgrowth probably by activating protein kinase A or adenylate cyclase in the PKA-dependent signaling pathway. In addition, PD98059 completely blocks the K vitamins-enhanced neurite outgrowth, demonstrating that K vitamins may also amplify an up-stream step of MAPK kinase. Sano *et al* (1998) reported that the activation of MAP kinases is not required for the NGF-induced extension of neurite from PC12D cells

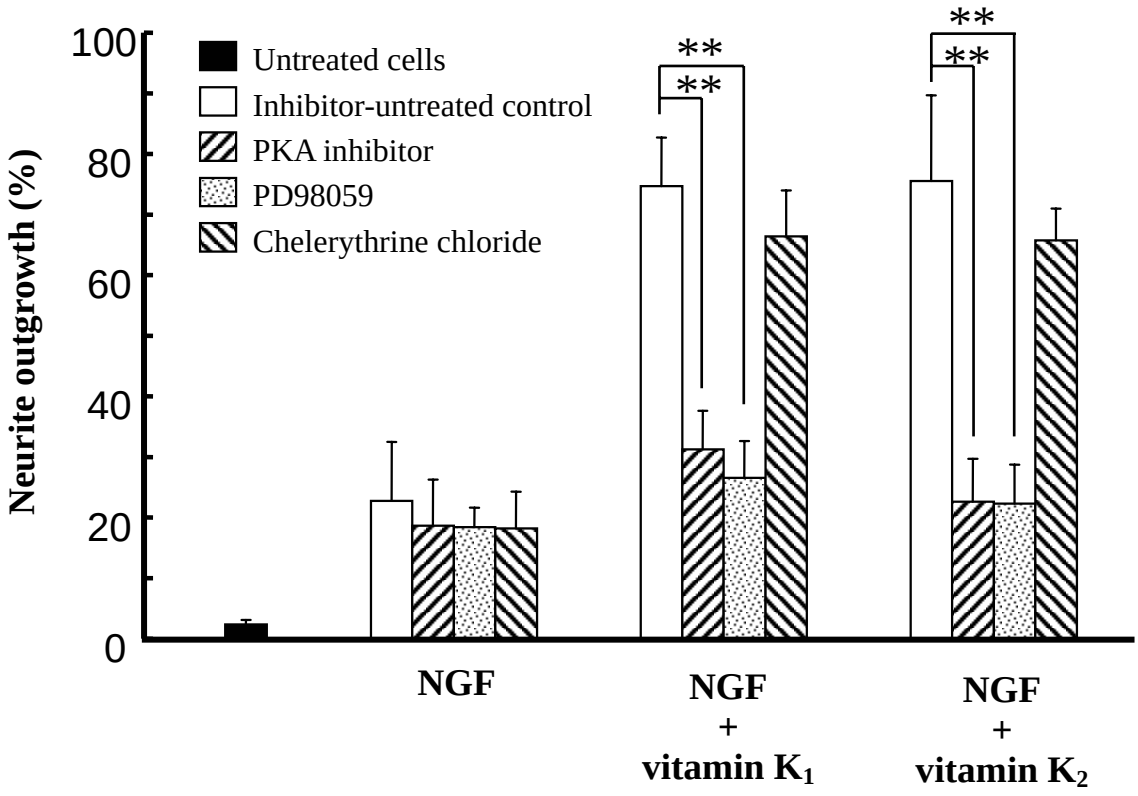


Fig. 9-4. Neurite outgrowth of PC12D cells in response to 10 ng/ml NGF, 10 ng/ml NGF plus 50 μg/ml K vitamins in the absence or presence of 5 nM PKA inhibitor, 10 μM PD98059 or 0.66 μM chelerythrine chloride. The neurite outgrowth is expressed as a percentage relative to the optimal response to NGF (50 ng/ml, 100%). Each point represents the mean ± SD (n=6) from two replicate experiments. Significant difference from the inhibitor-untreated control: **$P < 0.01$ (Student's *t*-test).

(Sano *et al*., 1998). Thus, K vitamins might activate MAP kinases as a separate signaling pathway, complementing the action of NGF in PC12D cells. However, it has been demonstrated that cyclic AMP induces neuronal differentiation via the activation of MAPK kinase in PC12 cells (Hansen *et al*., 2000). K vitamins might activate the cAMP-dependent PKA, subsequently activating MAPK kinase in PC12D cells. Although PKC's involvement in NGF-induced differentiation of PC12 cells has been suggested (Coleman & Wooten, 1994; Dupont *et al*., 2000), our data show that the PKC inhibitor chelerythrine chloride does not significantly block the effect of K vitamins, indicating that the action of K vitamins may be independent of PKC-mediated mechanism.

The present results suggest that K vitamins enhance neurite outgrowth from PC12D cells by the activation of PKA, subsequently amplifying the signal transduction via MAP kinases, while PKC is apparently not involved in the action of K vitamins.

It has been proposed that the sufficient daily uptake of vitamin K, especially for the elderly, may contribute to the prevention of risk of hip and spinal fractures (Suttie, 1991). As vitamin K is a relatively small molecule, they may rapidly enter the CNS after oral administration and might act in the CNS to enhance the effect of NGF on cholinergic basal forebrain neurons that are important in memory functions. The present study implies that the dietary intake of vitamin K or vitamin K-rich foods may also be beneficial to the protection of nervous system, and prevent or delay the onset of age-related pathogensis of neurodegenerative diseases such as Alzheimer's disease.

Chapter X

MC22, the Second NGF-Potentiating Substance Isolated from *Sargassum macrocarpum*

During a modified process for the extraction of MC14, another active fraction was identified from *S. macrocarpum*. Since this active substance was found to be more hydrophilic than MC14, a new chromatographic separation procedure was used for its purification. Finally, this active substance was successfully purified and designated as MC22. The chemical structure of MC22 was elucidated by NMR analyses. Its biological activities, including neurite outgrowth promoting effect and neuronal survival supporting effect, as well as the mechanisms will be described in this chapter.

10-1. Materials and Methods

10-1-1. Isolation and purification of MC22

The algal sample (500 g wet weight) of the brown alga *Sargassum macrocarpum* was washed 3 times with artificial sea water (ASW, Jamarine Laboratory), and once with phosphate-buffered saline (PBS). The sample was then cut into small pieces and homogenized in 2L PBS with a domestic mixer. The residue after centrifugation at 5000g was extracted with 2L methanol (MeOH) for 1 h by a mechanical homogenizer (Polytron, Kinematica) and the MeOH-extract was obtained after suction filtration through a #1 filter paper (Advantec). The filtrate of MeOH-extract was concentrated by

vacuum-evaporation to minimal volume, and then partitioned with 1L hexane-water-methanol (3:2:1) mixture. The fraction of aqueous methanol layer was further partitioned with chloroform-methanol-water (3:1:2). The chloroform fraction was subjected to the silica gel column chromatography (silica gel 60, Merck) (column size, ∅ 3.2 x 50 cm) eluting with chloroform-methanol (15:1). The flow rate was adjusted to 5 ml/min and a volume of approximately 5 ml per fraction was collected in glass tubes. The active fraction was determined by the bioassay of neurite outgrowth promoting activity as described in chapter II. Then, the pooled active fractions were subjected to a size exclusion chromatography (Toyopearl HW-40F, TOSOH) (column size, ∅ 3.2 x 50 cm1.7 x 50 cm) with 100% MeOH as eluting solvent. Active fractions were pooled, redissolved in hexane-ethyl acetate (6:4), and subjected to thin layer chromatography (TLC) (Silica gel 60, Merck) developing with hexane-ethyl acetate (6:4). The active fraction was collected at R_f value of 0.19-0.22, and finally purified by reverse phase high performance liquid chromatography (HPLC) using Shodex RS-pak DE-413 column (∅ 20 x 250 mm, Showa Denko) eluting with acetonitrile-water (9:1) at a flow rate of 3.5 ml/min and detecting at 212 nm by a UV detector (UV-970, Jasco).

10-1-2. Mass spectrometry

The molecular weight of MC22 was determined by a high resolution electron impact mass spectrometry (EI-MS, JMS-DX303, JEOL).

10-1-3. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectrometries

The chemical structure of MC22 was elucidated using ^{1}H - and ^{13}C-nuclear magnetic resonance spectra by Toray Research Center. The ^{1}H - and ^{13}C-NMR spectra of MC22

were recorded in CD_3OD by a GSX400 NMR spectrometer and a UNITY INOVA600 NMR spectrometer (Varian), respectively.

10-1-4. Neurite outgrowth promoting activity

The neurite outgrowth promoting activity of MC22 in various concentrations of NGF and the dose-dependent activity of MC22 were examined as described in section 4-2-3 and 4-2-4, respectively.

10-1-5. Neuronal survival promoting activity

The effect of MC22 on promoting survival of the neuronal PC12D cells was studied as described in section 6-1-1-(4) and 6-2-1-(1). The method used to investigate the mechanism of action of MC22 is described in section 6-1-1-5.

10-2. Results

10-2-1. Purification of MC22

The active fractions collected from various chromatographic columns during the purification of MC22 were traced by TLC (silica gel 60) with an R_f value of 0.35 (chloroform-methanol 15:1), and confirmed by bioassay of neurite outgrowth promoting activity. The active fractions on TLC plate was identified by their appearance of fluorescent blue color under UV illumination at 366 nm. Since MC22 was found to be more hydrophilic than MC14, it was purified by TLC developed with hexane-ethyl acetate (6:4) followed by a reverse phase chromatography with acetonitrile-water (9:1) as the eluting agent. The retention time of MC22, eluted with acetonitrile-water (9:1)

with a flow rate of 3.5 ml/min, was about 25 min. Finally, approximately 1.2 mg of MC22 was purified from 500 g wet weight of the alga. The absorption peaks of MC22 in methanol was observed at 220 nm (main) and 334 nm. MC22 showed pale yellow oil after freeze-dried and it was stored in methanol at 4°C for further bioassays.

10-2-2. Structural elucidation of MC22

Result of EI mass spectroscopic analysis revealed the molecular weight of MC22 to be 424. Based on various NMR spectra, MC22 was determined to be composed of five -CH_3 groups, six –CH_2- groups, one >C< group, seven =CH- groups, seven =C< groups, one –OH group, and one -COOH- group. Therefore, the molecular formula of MC22 was elucidated to be $C_{27}H_{36}O_4$. For the purpose of describing the structure of MC22 from the NMR spectra, peaks appeared in the ^{1}H-NMR spectrum were assigned with small letter a-q while peaks in ^{13}C-NMR spectrum were assigned with capital letters A-Z and Z'. The chemical shifts, proton number and multiplicity in both spectra are listed in Table 10-1. The results from HSQC spectrum (Fig. 10-1), together with the ^{1}H-NMR spectrum (Fig. 10-2) and ^{13}C-NMR spectrum (Fig. 10-3) indicated that CH_3 groups appeared at position (a,F), (b,B), (c,C), (e,E) and (h,A); CH_2 groups at (d,K), (f,J), (g,D), (g,G), (i,I) and (j,H); CH groups at (k,Q), (l,R), (m,T), (n,X) (o,P), (p,M) and (q,N). The splits of p and q (2.4 Hz) were resulted from the spin coupling between the protons at the meta-position of benzene structure. Results from the DQF-COSY spectrum (Fig. 10-4) revealed the partial structure of MC22 as shown in Fig. 10-5. The determination of c,e and the trans-configuration of structure in Fig. 10-5B were confirmed by NOESY

Table 10-1. Chemical shifts of ^{1}H- and ^{13}C-NMR spectra of MC22

^{1}H	Shift (ppm)	P.N.$^{\#}$	M (J)*	^{13}C	Shift (ppm)	M*
a	1.359	3	s	A	15.5	$-CH_3$
b	1.562	3	s	B	15.8	$-CH_3$
c	1.578	3	s	C	17.7	$-CH_3$
d	1.653	2	d,d (6.1,4.6)	D	22.6	$-CH_2-$
e	1.672	3	s	E	25.7	$-CH_3$
f	2.049	2	t (7.5)	F	26.0	$-CH_3$
g	2.110	4	m	G	27.8	$-CH_2-$
h	2.130	3	s	H	28.2	$-CH_2-$
i	2.250	2	t (7.5)	I	34.7	$-CH_2-$
j	2.551	2	q (7.5)	J	39.1	$-CH_2-$
k	5.084	1	t (7.10	K	40.7	$-CH_2-$
l	5.126	1	t (7.0)	L	77.8	>C<
m	5.569	1	d (9.8)	M	110.3	=CH-
n	5.926	1	br.t	N	117.1	=CH-
o	6.244	1	d (9.8)	O	121.4	=C<
p	6.319	1	d (2.4)	P	122.9	=CH-
q	6.742	1	d (2.4)	Q	123.5	=CH-
				R	124.8	=CH-
				S	126.4	=C<
				T	130.7	=CH-
				U	130.9	=C<
				V	132.2	=C<
				W	134.4	=C<
				X	144.3	=CH-
				Y	144.9	=C<
				Z	148.6	=C<
				Z’	171.5	>COO-

*Multiplicity: s-singlet, d-doublet, t-triplet, m-multiplet, br.-broad

$^{\#}$P.N. = proton number

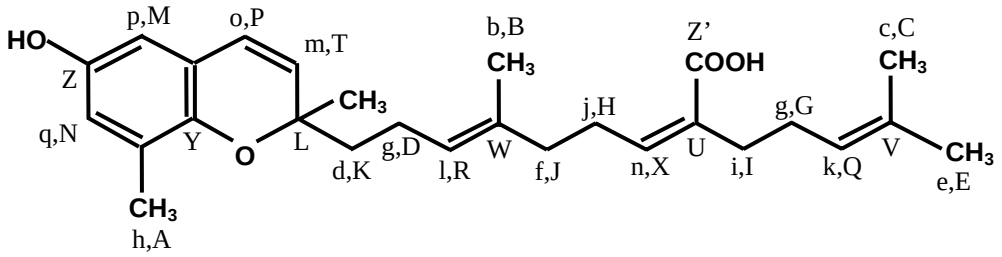


Fig. 10-1. HSQC spectrum of MC22.

Fig. 10-2. ^{1}H-NMR stectrum of MC22

Fig. 10-3. ^{13}C-NMR stectrum of MC22

Fig. 10-4. DQF-COSY spectrum of MC22

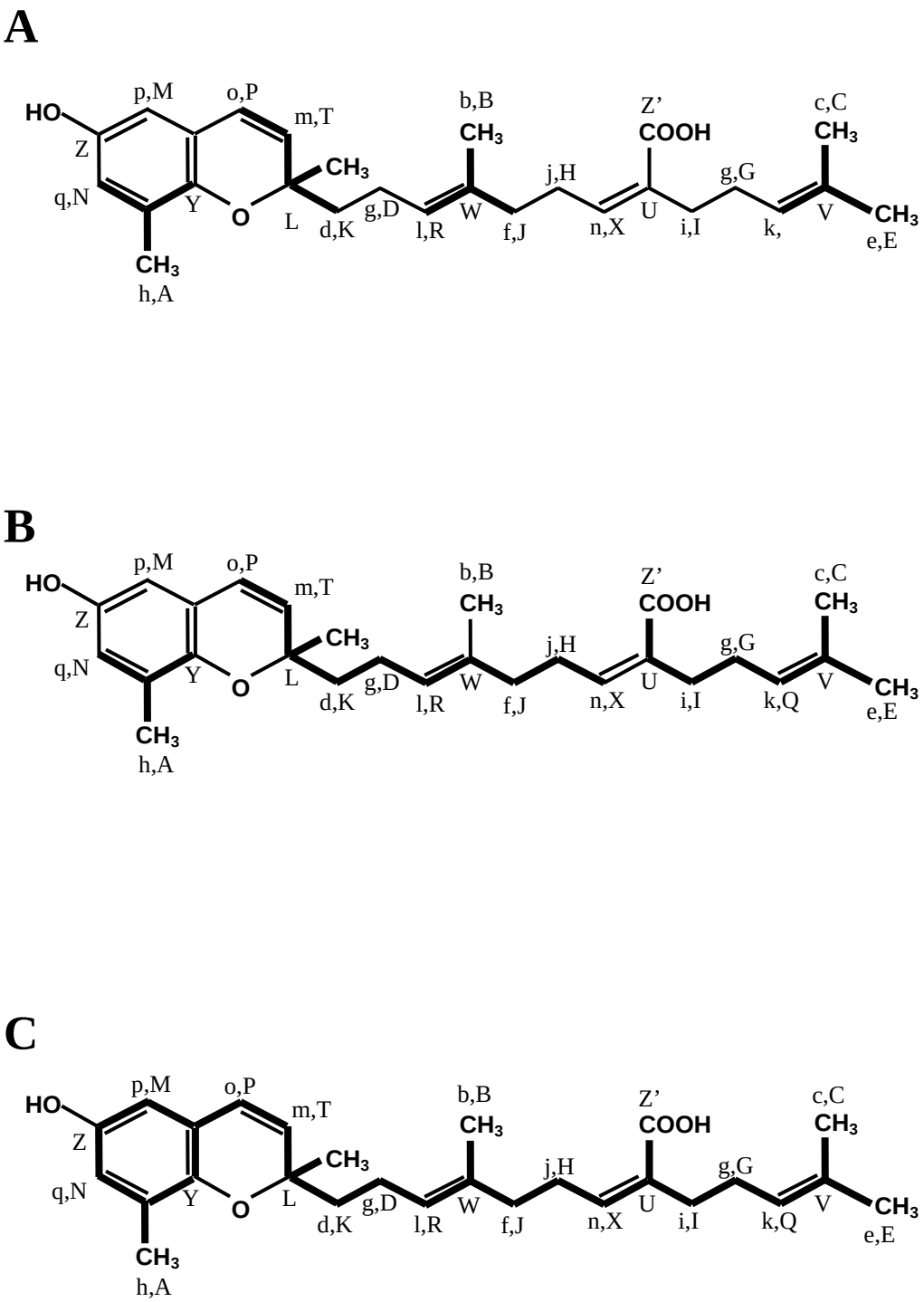
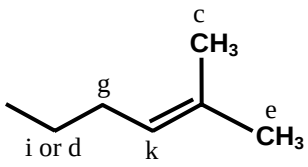


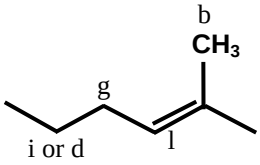
Fig. 10-5. Partial structures of MC22 determined by DQF-COSY, NOESY and HMBC.

Fig. 10-6. NOESY spectrum of MC22.

A



B



C

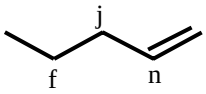


Fig. 10-7. Partial structures of MC22 determined by DQF-COSY, NOESY and HMBC.

Fig. 10-8. HMBC spectrum of MC22.

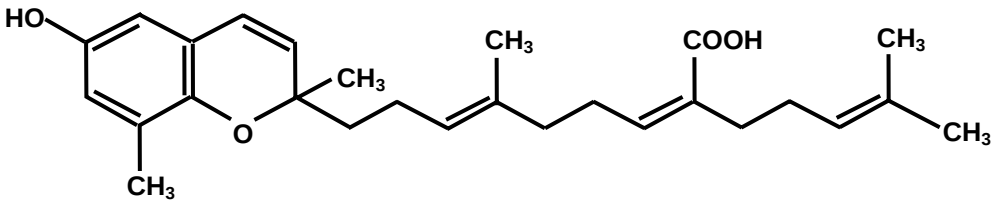


Fig. 10-9. Chemical structure of MC22

spectrum (fig. 10-6). The partial structures of carbon chain of MC22 shown in Fig. 10-7A was determined by the long-range couplings of the protons in CH_3 observed from the HMBC spectrum (Fig. 10-8). Accordingly, the conjugations of (d,D) and (d,R) to K-D-R, as well as (d,T) to L-T-P were confirmed. The conjugation of n-j-f was determined by DQF-COSY. Besides, The coupling of Z'-U-I-G-Q was determined by the related peaks of (i,U), (i,Q), (I,Z'). These data led to the identification of the partial structure of MC22 as shown in Fig. 10-7B. Based on the related peaks of m, o, p and q appeared in the HMBC spectrum, the partial structure of MC22 containing an 1,2-benzopyran structure was determined (Fig 10-7C). Together with the data obtained from NOESY spectrum, and the assignment of protons and carbons, the chemical structure of MC22 was elucidated and is shown in Fig. 10-9.

10-2-3. Neurite outgrowth promoting activity of MC22

The effect of MC22 on promoting neurite outgrowth from PC12D cells was examined from 0-100 ng/ml NGF. Treatment of PC12D cells with MC22 (12.5 μg/ml) significantly enhanced the proportion of neurite-bearing cells at 2.5-25 ng/ml NGF compared with the MC22-untreated control (Fig. 10-10). However, no significant neurite outgrowth was observed by the MC22-treatment alone. As shown in Fig. 10-11, the neurite outgrowth promoting activity of MC22 was dose-dependent. The maximum effect was observed when the cells were treated with 25 μg/ml MC22. Concentration of MC22 higher than 25 μg/ml resulted in cytotoxicity as cell shrinkage was observed.

10-2-4. Survival supporting activity of MC22

In order to evaluate the ability of MC22 to promote NGF-induced survival

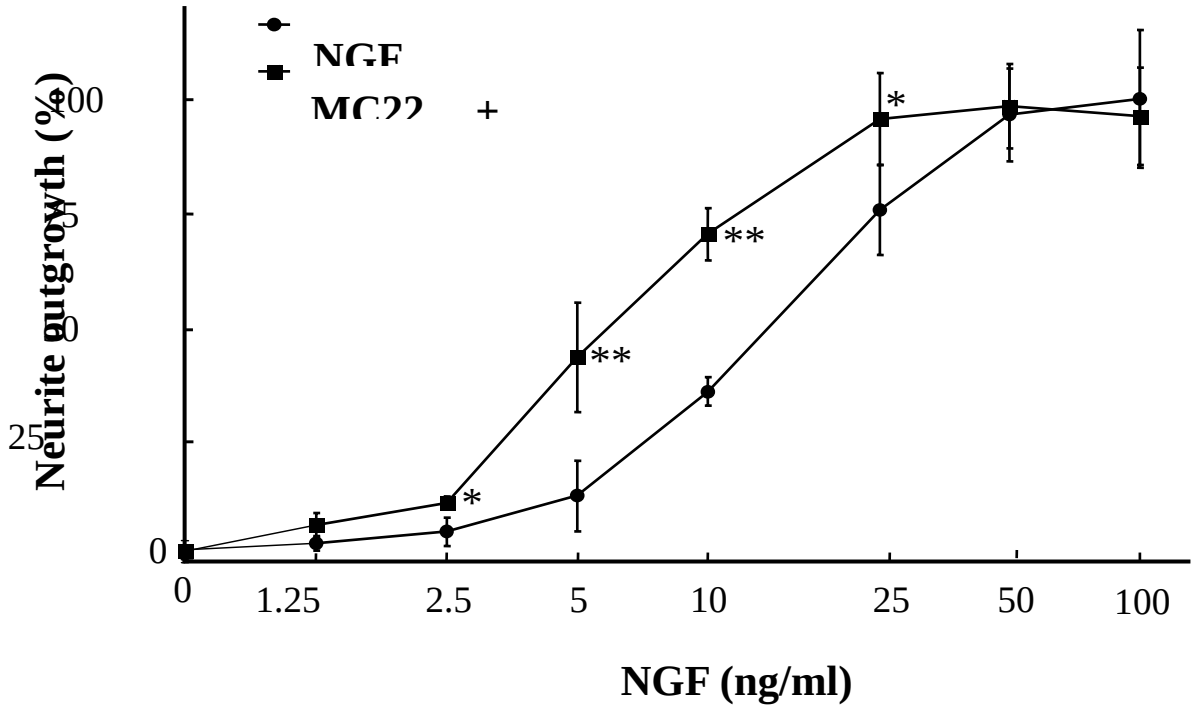


Fig. 10-10. Neurite outgrowth promoting activity of MC22 in the indicated concentrations of NGF. PC12D cells were treated with or without 12.5 μg/ml MC22 in NGF for 48 h. Each point represents the mean ± SD (n=4) from two replicate experiments. Neurite outgrowth is expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Significant difference from the NGF-only control: * *P* < 0.05; ** *P* < 0.01 (Student's *t*-test)

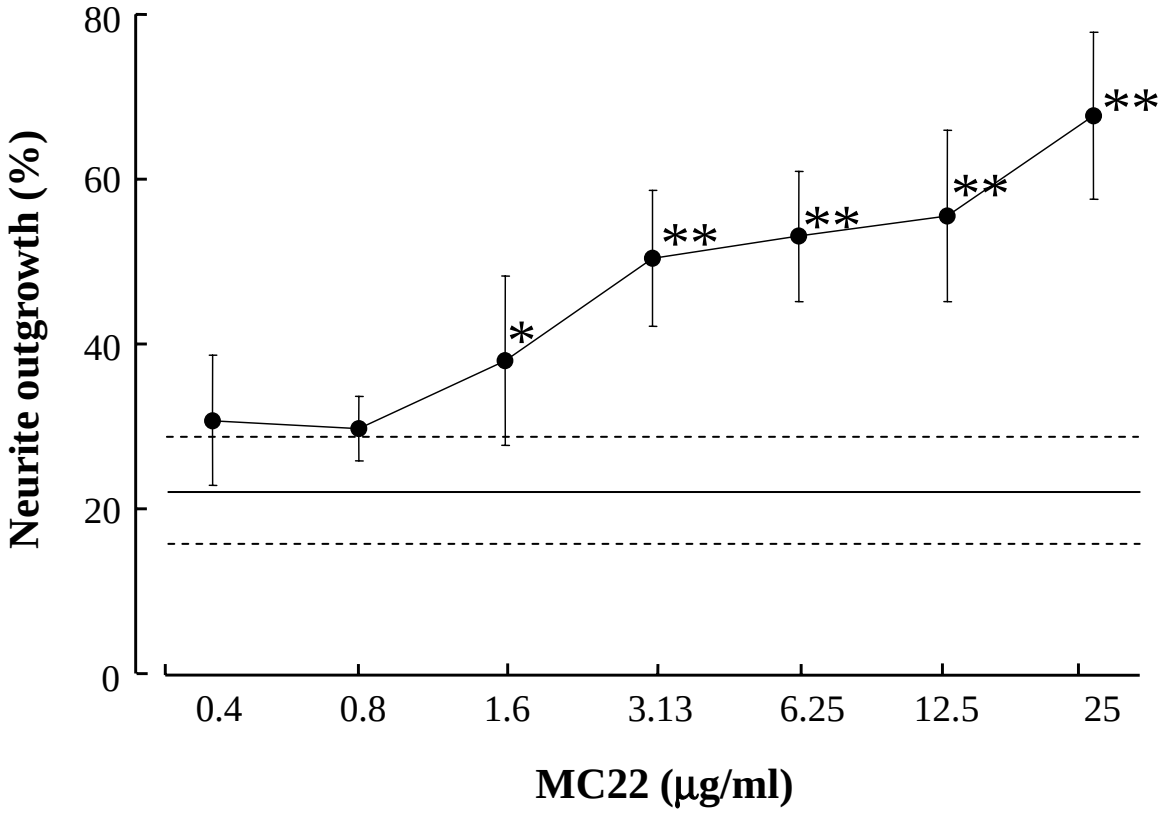


Fig. 10-11. Concentration-response curve of the neurite outgrowth enhancing effect of MC22 on PC12D cells in the presence of 10 ng/ml NGF. The horizontal solid line represents the mean value for cultures treated with 10 ng/ml NGF alone, the dotted lines represent the SD of this value. Neurite outgrowth is expressed as a percentage relative to the positive control (100 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4) from two replicate experiments. Significant difference from the NGF-only control: * *P* < 0.05; ** *P* < 0.01 (Student's *t*-test).

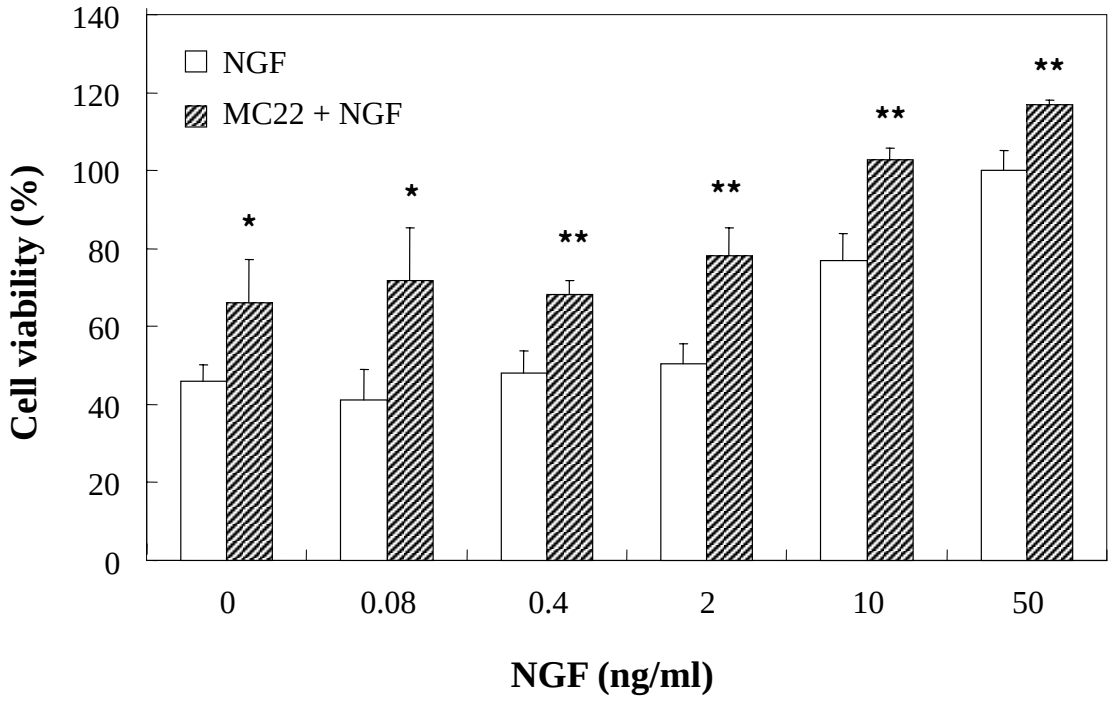


Fig. 10-12. Survival promoting effect of MC22 on neuronal PC12D cells. Neuronal PC12D cells were incubated in 4 μg/ml MC22 and the indicated concentrations of NGF in serum free medium for 24 h. Cell survival was determined by MTT assay, and expressed as a percentage relative to the positive control (50 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4). Significant difference from MC14-untreated control: * *P* < 0.05; ***P* < 0.01 (Student's *t*-test).

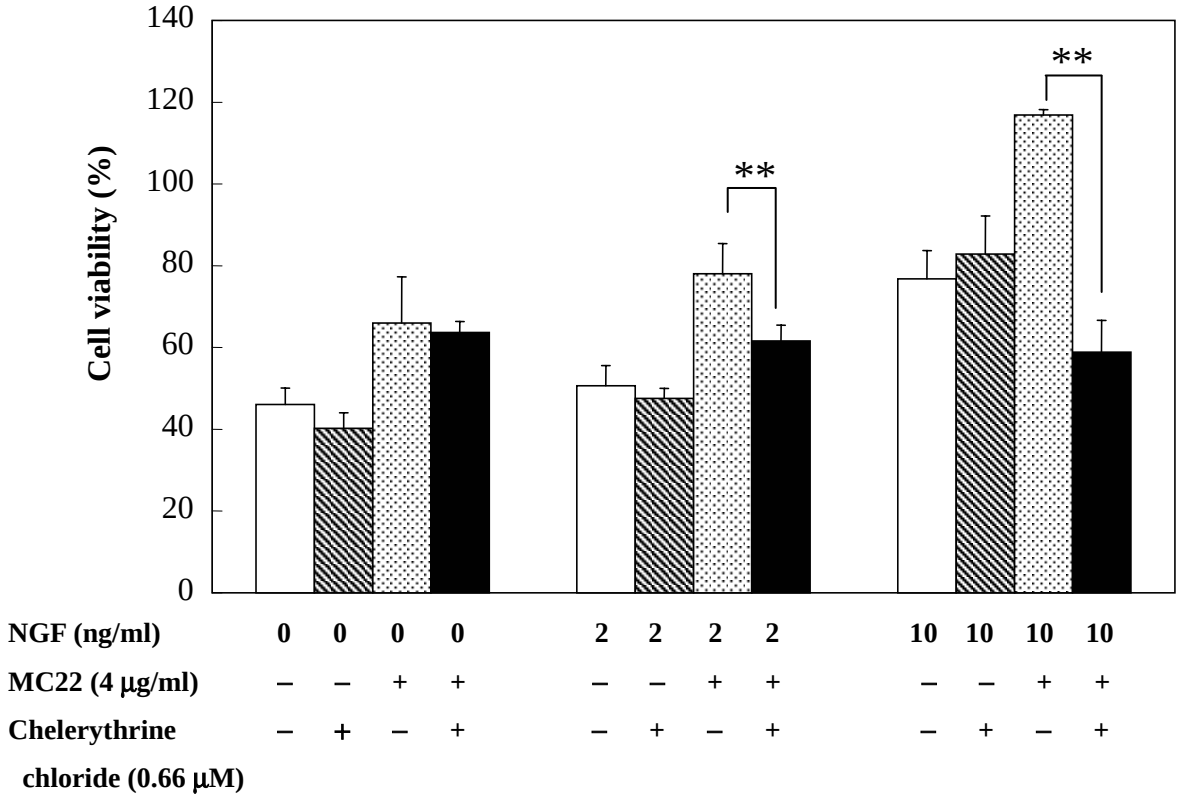


Fig. 10-13. Effect of chelerythrine chloride on survival promoting effect of NGF and MC22 on neuronal PC12D cells. Neuronal PC12D cells were pretreated with or without 0.66 μM chelerythrine chloride for 1 h before adding the indicated concentrations of NGF in serum free medium. After 24 h, cell viability was determined by MTT assay, and expressed as a percentage relative to the positive control (50 ng/ml NGF, 100%). Each point represents the mean ± SD (n=4). Significant difference from inhibitor-untreated control: **$P < 0.01$ (Student's *t*-test).

supporting effect, neuronal PC12D cells were treated with MC22 in the presence of various concentrations of NGF in serum-free medium. Cell viability was determined after 24 h incubation. Results showed that the treatment of MC22 significantly enhanced the number of viable cells at a concentration of NGF ranged from 0.08 to 50 ng/ml in serum-free medium (Fig. 10-12). Unexpectedly, MC22 alone exhibited appreciable rescuing effect on neuronal PC12D cells. Approximately 20% enhancement of viable cells was detected for cultures treated with 4 μg/ml MC22 compared with untreated control in serum-free medium.

To investigate the intracellular signaling pathway regulating the neuronal survival supporting effect of MC22, a PKC inhibitor, chelerythrine chloride was used to pretreat the cells before the addition of MC22 and/or NGF. Results indicated that chelerythrine chloride did not significantly block the survival supporting effect of either NGF or MC22 alone (Fig. 10-13). However, the inhibitor substantially block the combined effect of NGF and MC22, as approximately 10% and 40% reductions of viable cells were detected when the cells were treated with MC22, together with 2 ng/ml or 10 ng/ml NGF, respectively. This result suggested that PKC could be activated by the presence of both MC22 and NGF, while PKC activity was not affected by the individual treatment of MC22 or NGF in PC12D cells.

Regarding the neuroprotective effect of MC22 against H_2O_2-induced apoptosis on PC12D cells, the treatment of cells with 12.5 μg/ml MC22 in the presence of 0.4 and 2 ng/ml NGF significantly enhanced the number of viable cells incubated in 400 μM H_2O_2 compared with the NGF-only control (Fig. 10-14).

10-3. Discussion

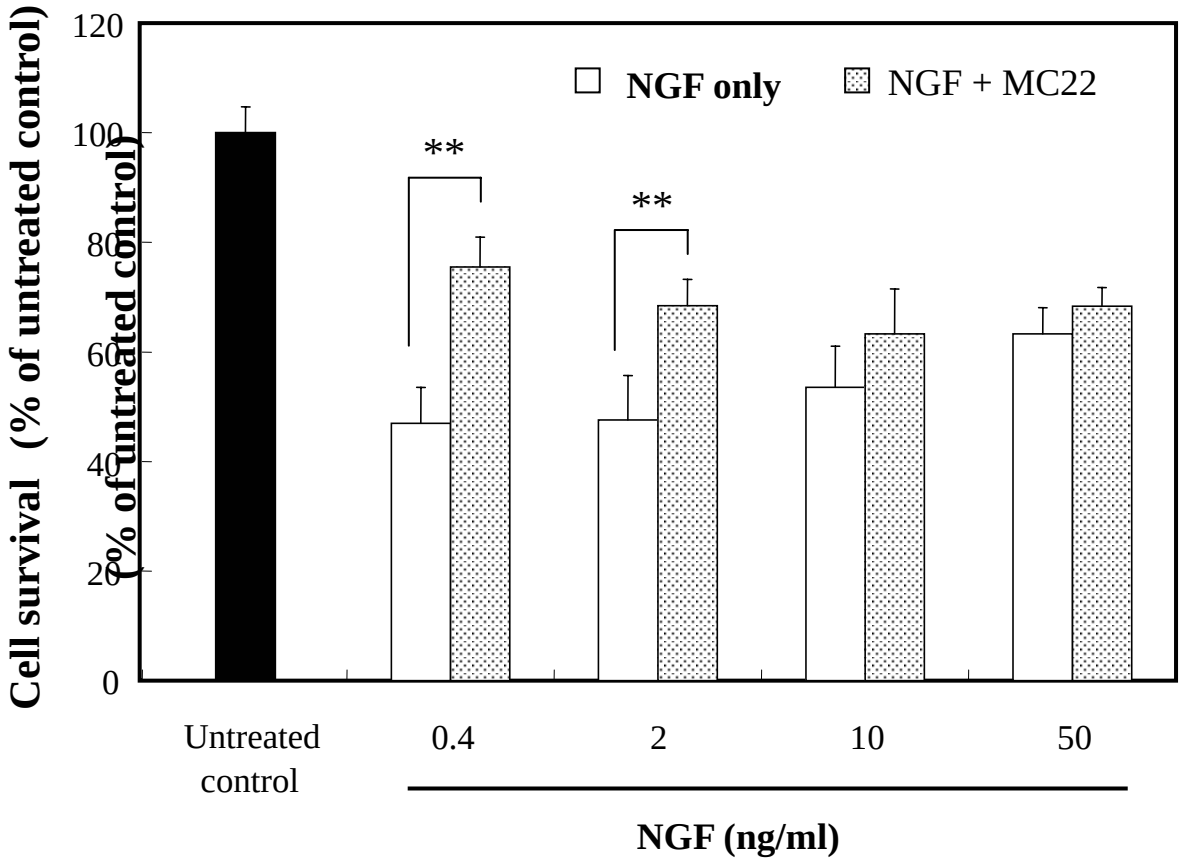


Fig. 10-14. Neuroprotective promoting effect of MC22 against H_2O_2-insult on PC12D cells. PC12D cells were pretreated with 12.5 μg/ml MC22 and the indicated concentrations of NGF for 2 h before the addition of 200 μM H_2O_2. After 6 h incubation, cell viability was determined by MTT assay, and expressed as a percentage relative to the untreated control (complete medium, 100%). Each point represents mean value ± SD (n=4). Significant difference from the corresponding NGF-only control: ** *P* < 0.01 (Student's *t*-test).

10-3-1. Purification and chemical structure determination

It is not unusual for the existence of more than one active metabolite in an organism. In fact, it is easy to overlook some of the other active fractions during the laboratory scale separation process of crude extract of an organism as these substances may be either ignored or undetectable in the bioassay (Cannell, 1998). During the isolation process of MC14 from *S. macrocarpum* with a modified extraction procedure, a fraction from the HPLC eluent was collected at a retention time of 22 min. This fraction was also found to have neurite outgrowth promoting activity on PC12D cells. As the retention time profile of this substance was different from that of MC14, confirming that it was the second active substance from the brown alga, *S. macrocarpum*. The HPLC elution profile revealed that this substance was more hydrophilic than MC14. Therefore, TLC and reverse phase HPLC were used to isolate this substance and finally it was successfully purified, and designated as MC22. By the NMR analyses, MC22 was elucidated to be sargachromenol with a molecular formula of $C_{27}H_{36}O_4$, indicating that MC22 is a non-peptide and low molecular weight NGF-potentiating compound. To evaluate its neural activity, the neurite outgrowth promoting activity and survival supporting activity of MC22 were examined.

10-3-2. Neural activity

In order to evaluate the neural activity of MC22, two typical NGF-mediated effects, neurite outgrowth promoting activity and neuronal survival promoting activity, were conducted. For the former, MC22 exhibits marked NGF-induced neurite outgrowth promoting activity on PC12D cells. Significant neurite outgrowth enhancement is also

observed at relative low concentration of NGF (2.5-10 ng/ml NGF). Besides, MC22 enhances neurite outgrowth in a dose-dependent manner, which is similar to that of MC14. However, MC22 alone does not induce neurite outgrowth on PC12D cells in the absence of NGF.

In order to examine the neuronal survival promoting activity of MC22, the neuronal PC12D cells were treated with NGF in the presence and absence of MC22 in serum-free medium. Result of cell viability measurement demonstrates that MC22 significantly increases the number of viable cells compared with the NGF-only control, suggesting that MC22 can effectively enhance the rescuing effect of NGF under serum-free condition. Surprisingly, MC22 can support the survival of neuronal PC12D cells in the absence of NGF in serum-free medium. It has been reported that several compounds can rescue PC12 cells from serum-free cell death but show no neurite outgrowth enhancing effect (Rukenstein *et al*., 1991). MC22 shows similar feature to those compounds. Besides, the present studies (chapters IV and VI), together with other reports in the literature, show the regulation of neuronal survival and neuronal differentiation may involve distinct signaling pathways (Rukenstein *et al*., 1991; Wooten *et al*., 1998; Hughes *et al*., 2001). To investigate the signaling pathway involved in MC22-enhanced neuronal survival activity, a protein kinase C inhibitor, chelerythrine chloride was examined. Results demonstrate that the survival supporting effect of either MC22 or NGF alone on neuronal PC12D cells is not blocked by PKC-inhibition, suggesting that MC22 and NGF promote survival on neuronal PC12D cells via PKC-independent pathway. Interestingly, the PKC inhibitor substantially blocks the combined effect of MC22 and NGF on neuronal survival action in serum-free medium, implying that PKC can be activated in the presence of both NGF and MC22, leading to survival support

action on neuronal PC12D cells. Although the exact mechanism of their additive action remains to be elucidated, it is postulated that the interaction of MC22 and NGF stimulates and activates the PKC-mediated pathway. In contrast, the combined effect of MC22 and NGF on neurite outgrowth from PC12D cells does not require the activation of PKC (data not shown). Taken together, these results confirm that MC22 regulates NGF-induced cellular responses via separate signaling cascades.

Concerning the neuroprotective effect against H_2O_2-induced apoptotic cell death, MC22 significantly enhances NGF-induced neuroprotective activity against oxidative stress at a relatively low concentration of NGF, although no significant effect can be observed at high concentration of NGF.

The present results show that MC22, the second active substance isolated from *S. macrocarpum*, is a low molecular weight and non-peptide compound that can significantly promote neuronal survival on PC12D cells, implying that MC22, like MC14, has therapeutic value for the treatment of neurodegenerative diseases.

General Conclusion

This doctoral research work can be concluded into four major findings from the following studies: (1) the successful purification and chemical elucidation of two novel neural active substances, MC14 and MC22, from the marine brown alga *Sargassum macrocarpum*; (2) the delineation of the strcuture-activity relationship of MC14; (3) the comprehensive investigation of the neural activities of MC14 and MC22 for the evaluation of their potential to be a therapeutic agent to treat neurodegenerative diseases; (4) the elucidation of signaling pathways regulating the effects of MC14 and MC22.

Previous screening works of the marine algae collected from Japan coastline had identified a brown alga, *S. macrocarpum* contains a neural active substance (designated as MC14), which showed potent neurite outgrowth promoting activity. In this doctoral study, MC14 has been purified and its chemical structure was successfully elucidated to be sargaquinoic acid, with a molecular weight of 424. Moreover, during a modified isolation process of MC14, another neural active substance was identified and successfully purified using various chromatographies. The chemical structure of this substance was finally elucidated as sargachromenol, and designated to be MC22.

The structure-activity relationship study demonstrates that the quinone moiety of MC14 structure plays a crucial role in its neuritogenic activity, and the structural modification of substitute group on the quinone moiety may enhance the activity of MC14 as the bonding of a hydroxyl group at the 1’-position of the benzoquinone moiety, or two hydroxy groups at the 1’ and 2’-positions of anthraquinone shows the enhancing neurite outgrowth promoting activity.

With respect to the biological activities, MC14 and MC22 markedly enhance NGF-induced neurite outgrowth from PC12D cells in a dose-dependent manner, clearly demonstrating that they possesses the neuronal differentiation promoting activity. In addition, the chronic effect of MC14 is shown to promote the network-like formation of neuritic processes, further supporting that MC14 may be beneficial to the development of the nervous system. Apart from the morphological differentiation, the treatment of MC14 significantly increases acetylcholinesterase activity, an important enzyme for maintaining the neurotransmitter function in neuronal cells, in the PC12D cells, suggesting that MC14 can also promote functional differentiation.

In addition to neural differentiation, NGF has an important role to support the survival of many neuronal populations of the central and pheripheral nervous systems, and the deficit of NGF in the nervous system has been linked to the pathology of many neurodegenerative diseases. One of the most encouraging findings in this study is that MC14 and MC22 can significantly protect neuronal PC12D cells against serum-free induced cell death. The important implication of this finding is that MC14 and MC22 may rescue neuron cells or slow down the rate of degenerative neuronal loss caused by Alzheimer’s disease or other neuodegenerative disorders. In addition to promote NGF-induced survival support, strikingly, MC14 and MC22 also exhibit neuronal survival supporting effect even in the absence of NGF. This finding, together with the neuroprotective effect of MC14 and MC22 on PC12D cells against oxidative stress-induced cellular damage, further implies that MC14 and MC22 may be effective in alleviating neuronal cell death caused by other neurotrophic factor-independent neurodegenerative diseases, such as Parkinson’s diseases or Huntington’s disease. On the other hand, neuroregeneration study provides supporting evidence for the capability

of MC14 to regenerate damaging neurites, implying that MC14 may help repairing the degenerating neuronal cells by promoting the reconnection of synaptic system.

Concerning the mechanisms of MC14 to potentiate NGF-induced neurite outgrowth from PC12D cells, several intracellular signaling cascades were shown to involve in the regulation of the observed effect of MC14. First, the activation of TrkA receptor is required for the NGF-induced neurite outgrowth from PC12D cells. MC14 may facilitate the binding of NGF into its receptor TrkA and in turn promote the activation of TrkA to amplify the signaling pathway. Besides, the activation of mitogen-activated protein kinase (MAPK) is essential for MC14-enhanced neurite outgrowth. Therefore, the TrkA-MAPK-mediating pathway is identified to be a central signal transduction cascade for regulating neurite outgrowth of PC12D cells in response to the stimulation of MC14 and NGF. Furthermore, PKA appears to be activated by MC14 during the neurite outgrowth response of PC12D cells. As the activation of PKA is independent of the TrkA-mediated cascade, these findings demonstrate that at least two distinct signaling pathways are activated in PC12D cells in response to MC14 stimulation during the NGF-induced neurite outgrowth. These two pathways may be activated by MC14 individually to complement the action of NGF. In addition, it is also inferred that MAP kinase might be a common downstream effector sheared by both PKA-mediated and TrkA-mediated signaling pathways to modulate the cellular response of PC12D cells to MC14 and NGF. These findings would contribute to the pharmacological manipulation of intracellular signaling pathway to potentiate the action of NGF.

Although the mechanism of MC14 to support survival of neuronal PC12D cells in NGF-deprived serum-free medium still remains to be elucidated, pharmacological inhibition study has revealed that the activation of PKA and MAP kinases are apparently

not required for the neuronal survival supporting activity of MC14, while the activation of other intracellular protein kinase is involved in the neuroprotective effect of MC14. This result demonstrates that MC14 employs separate signaling pathways to regulate neurite outgrowth and support survival in PC12D cells.

Since the delivery problem of neurotrophic factors into the brain is the largest obstacle for their development as effective therapy for neurodegenerative disorders. The neural active substances isolated from *S. macrocarpum*, MC14 and MC22, are samll molecules with molecular weight < 500 Da, and they are lipid-soluble, implying that these active compounds can cross the blood-brain barrier, and they may have promising pharmacological potential as an alternative strategy for the neurotrophic therapy. Furthermore, rescue of degenerating neurons as well as stimulation of neurogenesis are theoretically very attractive strategies for the treatment of many neurodegenerative diseases. At present, the available drugs for AD is limited to cholinesterase inhibitor, it would be very important to evaluate drugs with different mechanisms of action for their potential therapeutic application to this multifactorial disease. The findings in this study demonstrate that MC14 and MC22 can potentiate the action of NGF, including neurodifferentiation activity, neuroprotective activity against oxidative stress, and most importantly, the survival supporting activity and neurite-regenerating activities. These evidence strongly support the pharmacological application of MC14 and MC22 for the treatment of neurodegenerative disorders such as Alzheimer’ disease.

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